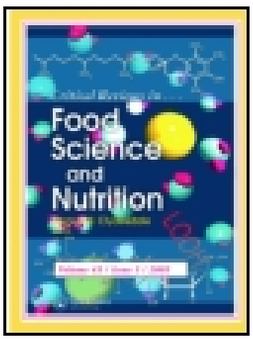

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REVIEW

The potential application of supercritical CO_2 in microbial inactivation of food raw materials and products

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ABSTRACT

The purpose of this study was to review the possibility of using supercritical CO_2 as a green and sustainable technology for microbial inactivation of raw material for further application in the food industry. The history of the development of supercritical CO_2 microbial inactivation has been widely described in this article. The fundamental scientific part of the process like mechanism of bactericidal action of CO_2 or inactivation of key enzymes were characterized in detail. In summary, this study provides an overview of the latest literature on the use of supercritical carbon dioxide in microbial inactivation of food raw materials and products.

KEYWORDS

Bacteria; microbial inactivation; microbiological contamination; supercritical CO_2; virus

Introduction

Microbiological quality and safety of food is currently an extremely important issue in the food industry. Modern preservation methods should prevent the development of spoilage and pathogenic microorganisms and ensure consumer safety. Preservation methods can be divided into physical and chemical (Figure 1). Thermal preservation of food is the most common technique for reducing the microbial contamination, however, its use can pose some problems such as excessive heating (deterioration of the organoleptic and sensory properties of food) or insufficient heating (insufficiently sterilized food products). For heat sensitive food, thermal pasteurization can introduce unwanted organoleptic changes that have negative effect on quality of food. Due to the increase in consumer demand for nutritious, fresh food products with high organoleptic qualities and extended shelf life, scientific teams proposed other non-thermal methods for processing products (Figure 1).

Among nonthermal technologies for inactivation of microbiological activity, high hydrostatic pressure (HHP) and pulsed electric fields (PEF) are the most studied (Devlieghere, Vermeiren, and Debevere 2004). In particular, the HHP method is considered as an alternative to microbial inactivation of foods, while retaining the initial composition and organoleptic parameters. Despite the fact that the method of high hydrostatic process offers excellent opportunities for preserving food ingredients, it also has some serious limitations, like an emergence of pressure-resistant vegetative bacteria after pressure treatment; the large investment costs or lack of process continuity for solid food products (Estrada-Girón, Swanson, and Barbosa-Cánovas 2005; Meloni 2019). These shortcomings hamper the large-scale implementation of the HHP method in food preservation processes in the food industry. Chemical methods of food preservation have also many drawbacks. There is a suspicion that they are the cause of many allergies and may also cause cancer.

In contrast to the above method, for almost two decades, the use of supercritical carbon dioxide has been proposed as an alternative technique of nonthermal microbial inactivation of food products (Garcia-Gonzalez et al. 2007; Spilimbergo, Elvassore, and Bertucco 2002). The number of published journal articles pertaining to microbial inactivation of food products using supercritical CO_2 ($scCO_2$) has increased significantly over the past 10 years. In this method, the food is contacted with sub- or $scCO_2$, depending on the process parameters, mainly pressure and temperature. $ScCO_2$ is a fluid at a temperature and pressure above the critical point ($T_c = 31.1°C$, $P_c = 7.38$ MPa) and then it has a unique ability to diffuse through solid particles, behaving like a gas, and dissolve chemical compounds, as a liquid. It is possible to easily change the value of density, viscosity,

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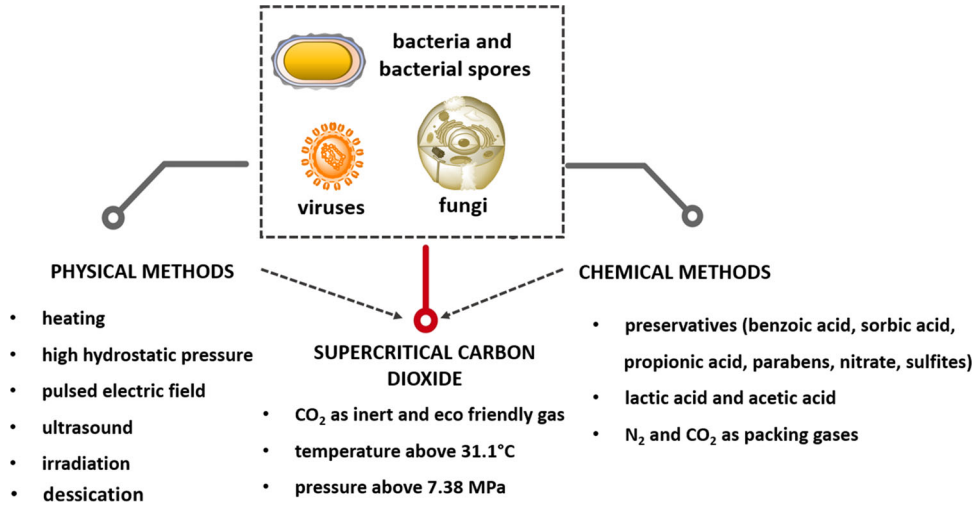

Figure 1. Methods of food preservation.

and surface tension of $scCO_2$ with insignificant changes in temperature or pressure values. Small changes in these parameters can result in sizeable changes in microbial inactivation efficiency (Wrona et al. 2017, 2018, 2019a). Moreover, $scCO_2$ is characterized by very low viscosity and almost zero surface tension, which greatly facilitates penetration of complex structures and porous materials like bacterial cells (Wrona et al. 2019a; Zhang, Burrows, et al. 2006; Soares et al. 2019).

$ScCO_2$ technology offers great advantages over other inactivation methods associated with milder conditions of use. The CO_2 pressure for conservation and microbial inactivation is significantly lower (typically <20 MPa) compared to the hydrostatic pressure used in the HHP method (~ 600 MPa). Moreover, CO_2 is not expensive, inert, nonflammable, and approved by the European Food Safety Authority (EFSA) for use in various industries. Microbial inactivation with $scCO_2$ is in line with the assumptions of green technology. Although CO_2 is considered a greenhouse gas, modern technologies allow it to be post-processed and reused, which significantly reduces environmental emissions (Soares et al. 2019; Dillow et al. 1999).

The possibility of using $scCO_2$ as a sterilizing agent for the first time was examined by Kobayashi and his group in 1987 (Soares et al. 2019; Kamihira, Taniguchi, and Kobayashi 1987). They proved antibacterial effect of this technique on *Escherichia coli*, *Staphylococcus aureus* and conidia of *Aspergillus niger* at pressure 20.3 MPa and temperature 35 °C. The content of water was important limiting factor. The parameters used were only effective for cells containing 70%–90% water, while cells with a water content of 2%–10% were resistant (Kamihira, Taniguchi, and Kobayashi 1987). Numerous literature data indicate that vegetative bacteria can be fully inactivated with $scCO_2$ under relatively low parameters of pressure and temperature and without any additive. In some laboratory tests, at least a 6-log reduction in the number of the colony-forming unit (CFU) after $scCO_2$ treatment at 35 °C was observed for *Salmonella typhimurium*, *Escherichia coli*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Aeromonas hydrophila*, *Salmonella enteritidis*, *Yersinia enterocolitica*, and *Staphylococcus aureus* suspended in physiological saline or phosphate-buffered saline (Kim et al. 2007, 2008; Furukawa et al. 2009). Furukawa et al. (2009) reported 6-log reduction of nine foodborne bacteria (*Pseudomonas aeruginosa*, *Aeromonas hydrophila*, *Salmonella enteritidis*, *Salmonella typhimurium*, *Yersinia enterocolitica*, two strains of *Escherichia coli*, *Staphylococcus aureus*, and *Listeria monocytogenes*) after 1 min of exposure to 10.0 MPa scCO2 at 35 °C. However, serious limitation of this method is high resistance of dormant forms—endospores.

The literature has many examples of the use of $scCO_2$ in food stabilization in laboratory research. Recently, it has been shown that $scCO_2$ is effective in inactivation of *Staphylococcus aureus* in raw salmon (Cuppini et al. 2017). For this method, the level of bacterial reduction in raw and skim milk was comparable to high temperature, short-time pasteurization (Werner and Hotchkiss 2006). The inactivation capacity of $scCO_2$ was also confirmed for *Escherichia coli*, *Salmonella*, and *Listeria monocytogenes* during apple's slices drying (Zambon et al. 2019). Despite the increase in research over the past ten years, the $scCO_2$ treatment technology for microbial inactivation of products has not yet been widely introduced in the food industry.

The mechanism of bactericidal action of carbon dioxide

Valley and Rettger almost 100 years ago discovered that CO_2 has an inhibitory effect on bacterial growth (Garcia-Gonzalez et al. 2007; Valley and Rettger 1927). Since then, many scientific articles have been published on the study of the effect of CO_2 on the growth of microbial and the positive impact of $scCO_2$ on the quality of food. The data published so far show that vegetative forms of bacteria are fully susceptible to inactivation with the $scCO_2$ technology under mild conditions of pressure and temperature. For a long time, the specific mechanism of the bacteriostatic effect of CO_2 was not known. In 1985, Daniels et al. analyzed the basic theory that explains the bacteriostatic effect of gaseous

CO_2 in their review (Daniels, Krishnamurthi, and Rizvi 1985).

The action of carbon dioxide on bacterial cells occurs subsequently on many levels and involves such processes as solubilization of compressed CO_2 in the liquid phase; disintegration of cell membrane, inactivation of enzymes, disruption of electrolyte balance, and extraction of cellular components from cell membranes and cytoplasm (Garcia-Gonzalez et al. 2007).

Carbon dioxide dissolves in water to form carboxylic acid (H_2CO_3), which dissociates into bicarbonate (HCO_3^-), carbonate (CO_3^{2-}) and hydrogen (H^+) (Duan and Sun 2003). The mentioned reactions can be described by the following chemical equations:

$$CO_2(g) \rightleftharpoons CO_2(aq) \quad (1)$$

$$CO_2(aq) + H_2O \rightleftharpoons H_2CO_3 \quad (2)$$

$$H_2CO_3 \rightleftharpoons H^+ + HCO_3^- \quad (3)$$

$$HCO_3^- \rightleftharpoons H^+ + CO_3^{2-} \quad (4)$$

The same reaction occurs in food with high water content. Studies indicate that the dissolution of CO_2 in water is crucial because low-water bacterial cells are not susceptible to inactivation with carbon dioxide (Dillow et al. 1999; Kamihira, Taniguchi, and Kobayashi 1987). Water, interacting with pressurized CO_2, usually becomes acidic due to the formation and dissociation of H_2CO_3, which releases H^+ ions. This lowered extracellular pH (pH_{ex}) significantly reduce microbial growth. One of the reason is the reduction in the resistance of the microbiota to inactivation due to the extreme increase in energy costs to maintain pH homeostasis by proton-translocating ATPase—enzyme that uses energy from ATP to transfer H^+ (Hutkins and Nannen 1993). However, lowering the pH is not sufficient to explain the lethal effect of CO_2 on microbial cells, because carbon dioxide has a much greater inhibitory effect than hydrochloric acid or phosphoric acid at the same pH (Lin, Cao, and Chen 1994). Moreover, it was evidenced that carbon dioxide had inhibitory effect on bacterial cell even in the presence of buffer (Wan et al. 2016), which clearly suggests that not only low pH is the reason for cell damage. However, the permeability of bacterial cells increases with decreasing pH, which facilitates the penetration of CO_2 through the cell walls.

The aqueous (non-hydrated) CO_2 can diffuse into membranes and accumulate in the lipophilic inner layer. Hutkins and Nannen (1993) suggested that CO_2 dissolves well in phospholipids of the cell membrane, which is also related to the non-polar nature of both CO_2 and phospholipids (Garcia-Gonzalez et al. 2007; Spilimbergo 2002). A large amount of CO_2 in the lipid layer can damage the function of the cell membrane due to loss of lipid chain order. This process is called "loss of sensitivity" or "anesthesia" and leads to an increase in membrane permeability (Garcia-Gonzalez et al. 2007; Jones and Greenfield 1982). Fluidization of the membrane caused by exposure to CO_2 (at pressure up to 13.9 MPa) was demonstrated on a sample of the cell membrane of a thermophilic bacteria *Clostridium thermocellum* (Bothun et al. 2005). On the other side, generated HCO_3^- ions can also interact with charged groups of phospholipids and proteins on the surface of the membrane and as a result, change in the surface charge occurs.

Supercritical CO_2 easily penetrates the membrane, as the permeability of the membrane is increased, and then accumulates in the cell cytoplasm. Intracellular pH (pH_i) in cells is strictly controlled. The membrane bound enzyme H^+–ATPase (adenosine triphosphatase) is the most important homeostatic system that displaces protons from the cytoplasm according to the difference in the pH gradient $\Delta pH = pH_i - pH_{ex}$, where pH_{ex} is extracellular pH and the electrochemical gradient (trans-membrane potential) (Hutkins and Nannen 1993). Cells cannot keep up to remove all the protons released during carboxylic acid dissociation and pH_i begins to decrease.

The catalytic activity of enzymes depends on pH_i value. Each enzyme possesses optimum pH at which exhibits maximal activity. Decreased cytosolic pH_i can cause inactivation of key enzymes necessary for metabolic and regulatory processes in the cell, such as glycolysis, peptide transfer, proton translocation, translation, replication, and active ion transport. For example, glycolysis that is the first step of cellular respiration is inhibited when the intracellular pH decreases below 3 (Spilimbergo 2002).

Upon exposure of *E. coli* to supercritical carbon dioxide, the activities of intracellular enzymes such as alkaline phosphatase, leucine arylamidase, acid phospatase, naphthol-AS-BI-phosphohydrolase, β-glucuronidase, and α-glucosidase initially increased and after 10 min continuously decreased (Kim et al. 2007). Watanabe et al. demonstrated that in *E. coli* cells treated with high pressure carbon dioxide internal pH of cells drops to 5.2 (Watanabe et al. 2007). A rapid drop in internal pH was also confirmed in pH-sensitive GFP-variant of *Saccharomyces cerevisiae* (Giulitti, Cinquemani, and Spilimbergo 2011). In these conditions, DNA becomes entangled in coagulated cytoplasmic proteins. The change in interaction between DNA and proteins can lead to inhibition of transcription (RNA synthesis) and replication (DNA synthesis) processes. Yao et al. during studies on membrane permeability with propidium iodide concluded that in low pH, the denaturation of DNA and unwind of double stranded helix can occur (Figure 2) (Yao et al. 2014). They indicate that nucleic acid denaturation can be one critical reason of inactivation of *E. coli* during treatment with $scCO_2$.

At low pH values changes in charge of different functional groups occurs. They arouse intensive electrostatic attraction and repulsion between side chains of proteins and as a result, changes in the folding of proteins appears. Even minor conformational modifications in structure of enzymes can cause changes in activity and specificity. On the other hand, CO_2 can form complexes with amino groups from histidine and lysine present on the surface of enzymes called carbamates (Figure 2). This reaction removes charges on the enzyme and contributes to the loss of activity (Fricks et al. 2006; Hu et al. 2013).

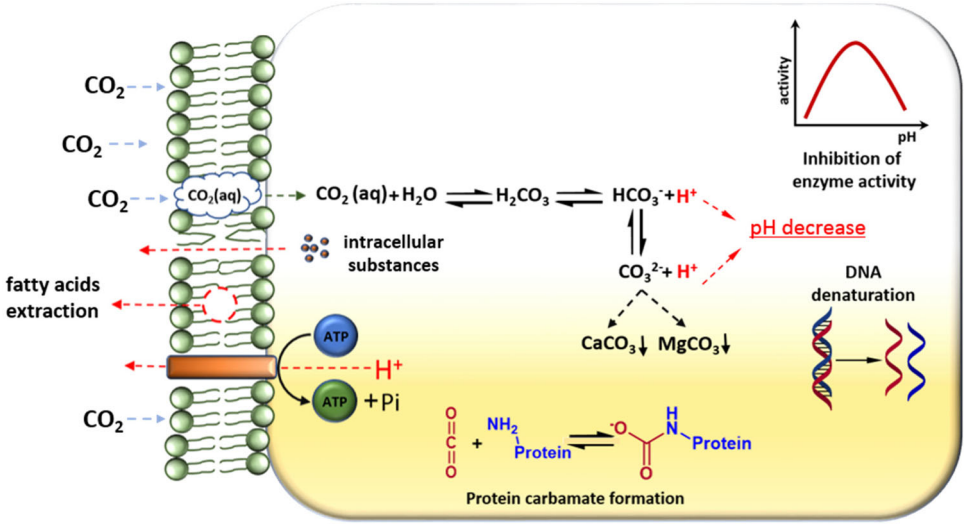

Figure 2. Schematic representation of the process, how $scCO_2$ can have a lethal effect on microorganisms (according to Garcia-Gonzalez et al. 2007, modified).

Regulation of metabolism occurs at many levels. The rate of each enzymatic reaction depends on the pH, on the intracellular concentration of its substrates and products. The concentration of the HCO_3^- anion, which is controlled intracellularly by internal pH buffering, is most likely to play a decisive role in regulating enzymatic activity and, therefore, cellular metabolism (Jones and Greenfield 1982). Regulation has an effect on the anion-sensitive region, which is present in the enzymes. Jones and Greenfield found the effect of the anion HCO_3^- and dissolved (unhydrated) CO_2 on the carboxylation and decarboxylation reactions (Jones and Greenfield 1982). In these reactions, CO_2 acts either as a biosynthetic substrate in the carboxylation reaction or in the decarboxylation reaction as metabolic product. Especially important are the reactions of carboxylation for the biosynthesis of glucose from non-carbohydrate substrates and the synthesis of specific biosynthetic amino acid precursors and nucleic acids. Since there is no generalized preference for either dissolved (unhydrated) CO_2 or HCO_3^- as a substrate in the carboxylation reactions, the ratio of dissolved CO_2/HCO_3^- will partially determine the relative reaction rate at which different carboxylase families are acting.

Regarding decarboxylation reactions, it is clear that dissolved CO_2 can inhibit these reactions. The inhibitory effect that CO_2 has on decarboxylases is not clear and can be explained either by inhibiting the product with CO_2, or by the equilibrium effect of "mass action" (Garcia-Gonzalez et al. 2007).

Lethal damage to the biological cell system can also occur when the pressure of the applied CO_2 is amplified in the cytoplasm of bacterial cells. This can turn HCO_3^- into CO_3^{2-}, which can precipitate intracellular inorganic electrolytes Ca^{2+}, Mg^{2+} (Lin, Yang, and Chen 1993). Since these inorganic electrolytes play an important role in maintaining osmotic bonds between cells and their environment, precipitation certainly has a detrimental effect on the entire cell. For example, the cytoplasmic concentration of free Ca^{2+} is several orders of magnitude smaller than its total intracellular concentration, which implies the enormous buffering of Ca^{2+} by intracellular components. Some types of these Ca^{2+} - and Mg^{2+} - binding proteins can also be available for precipitation with CO_3^{2-}, depending on the chemical structure of the protein (Garcia-Gonzalez 2007). In addition, a decrease in the proton motive force as a whole across the membrane due to a decrease in pH_{ex} can also lead to an imbalance in the cytoplasmic levels of Ca^{2+}. It is well established that calcium ions are cell regulator and are involved in the maintenance of cell structure, transport, and cell differentiation processes (Dominguez 2004). Therefore, changes in calcium gradient can lead to serious disturbances in the cell function.

Supercritical CO_2 is a great solvent for extraction of nonpolar compounds from different matrices hence it can be concluded that can "extract" vital components from bacterial cells or cell membranes due to its high solvating ability. During extraction intracellular components such as phospholipids and other hydrophobic compounds, disruption or alteration of the membrane structure can occur (Lin, Yang, and Chen 1992, 1993). An important parameter associated with the extraction process is mass transfer that can be enhanced by the sudden release of pressure.

Ballestra, Silva, and Cuq (1996) confirmed the above theoretical studies by experiments, they demonstrated that *E. coli* cells exposed to CO_2 at a pressure of 5 MPa and 35 °C showed some signs of deformation of the cell walls. Hong and Pyun showed that *L. plantarum* cells, under CO_2 pressure of 7 MPa, at 30 °C for 10 min acquire irreversible damage to the cell membrane, lose UV absorbing substances, and release intracellular ions Mg^{2+} and K^+ (Hong and Pyun 2001). In support of this hypothesis, it was discovered that intracellular enzymes of bacterial cells (*E. coli*) were removed to the extracellular environment after treatment with CO_2 (Bertoloni et al. 2006). Studies by Xu et al. (2017) showed changes in the profile of saturated and unsaturated fatty acids during treatment of *Vibrio parahaemolyticus* with $scCO_2$. The extraction effect of saturated fatty acids was more significant than unsaturated fatty acids. The reason for the decrease in saturated fatty acids was probably deletion of lipid A. Analyzing the above studies, we can conclude that, of course, deformation and damage to the integrity of the

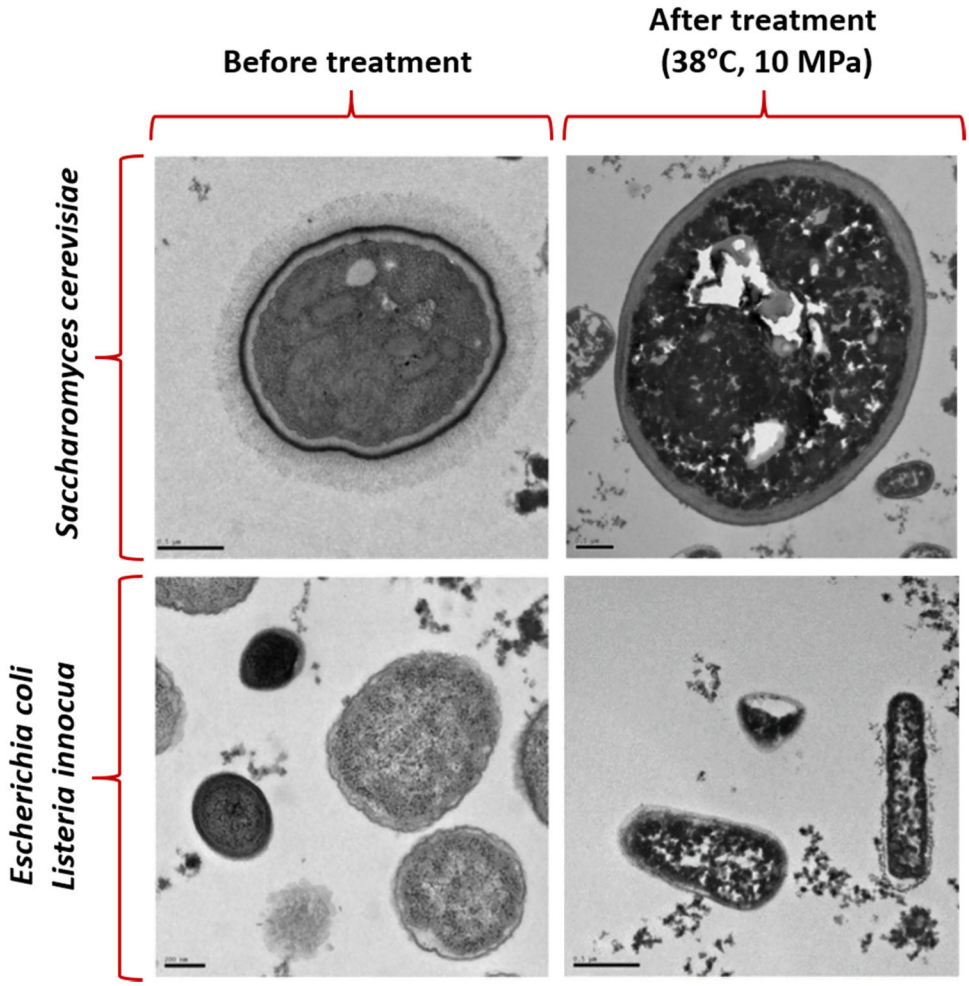

Figure 3. Ultrastructure of *Saccharomyces cerevisiae*, *Escherichia coli* and *Listeria innocua* before and after treatment with $scCO_2$. Reprinted and modified from (Fleury et al. 2018).

cell membrane can be the cause of the death of some of the cells but we cannot exclude that also other destructive mechanisms contribute to the lethality of $scCO_2$ and, most likely, have a synergistic effect (Figure 3) (Fleury et al. 2018).

Mechanism of bacterial spores inactivation

Endospore is dormant and non-reproductive form of some bacteria from *Firmicutes phylum*. Spores are extremely resistant to ultraviolet radiation, desiccation, high temperature, extreme freezing and harsh chemicals, free radicals, and radiation. They allow bacteria to survive in an adverse environment for a very long time. Examples of bacteria that can form spores and are important from the point of view of the food industry are *Bacillus cereus*, *Clostridium perfringens*, and *Clostridium botulinum*. These spore-forming bacteria are responsible for food spoilage as well food borne disease and lead to substantial economic losses in the food chain and to substantial food waste (Checinska, Paszczynski, and Burbank 2015; Postollec et al. 2012; Stecchini, Del Torre, and Polese 2013). Since spores are particularly resistant to $scCO_2$, hence a lot of research currently focuses on the development of this method to be effective against these dormant forms (Lopes et al. 2018; Rao, Bi, et al. 2016). Research conducted on the use of scCO2 to sterilize medical device has shown that the effectiveness of the process can be increased by introducing additives such as H_2O_2 or peracetic acid. However, due to the toxicity of these solutions, it is not possible to introduce them into microbial inactivation in food industry.

It has been proposed the mechanism in which endospores firstly germinate into vegetative forms, and then they are inactivated as normal vegetative forms (Spilimbergo, Elvassore, and Bertucco 2002; Zhang, Burrows, et al. 2006; Spilimbergo et al. 2003; Watanabe et al. 2003). However, later studies showed that germination of spores did not occur under $scCO_2$ which contradicts earlier assumptions and indicate that mechanism of spore inactivation is different (Rao, Bi, et al. 2016). It was evidenced that high pressure CO_2 induces structural changes in the inner membrane of spores (Rao, Bi, et al. 2016; Zhang, Burrows, et al. 2006; Setlow et al. 2016; Zhang et al. 2007) which is associated with increased permeability of both inner membrane and cortex. Scanning electron microscopy analysis showed cell debris and deformed spores after process. However, transmission electron microscopy confirmed damage in cortex and internal membrane as well deformed coat (Figure 4). Based on these results, it is postulated that high pressure

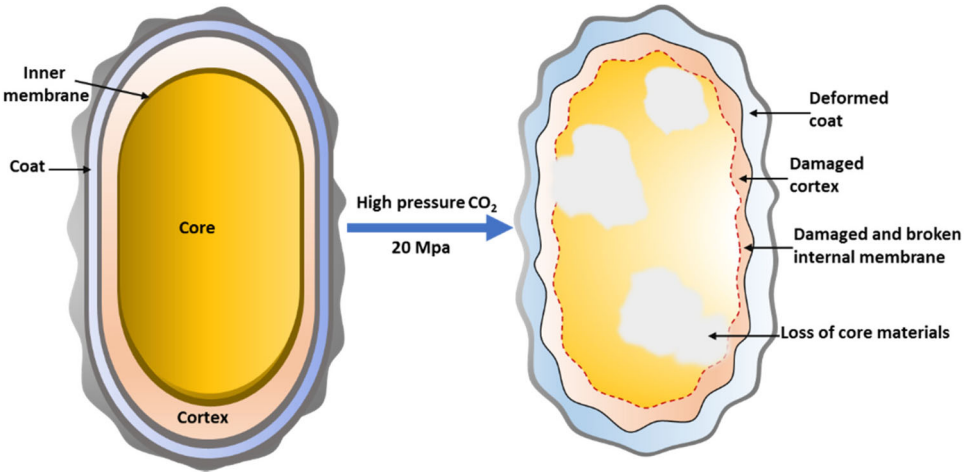

Figure 4. Mechanism of bacterial endospores inactivation by high pressure CO_2, based on the results obtained by (Rao, Bi, et al. 2016).

CO_2 leads to increased permeability of internal membrane and finally to damage proteins associated with spore germination which are located in the inner membrane. These changes prevent further spore germination (Rao, Bi, et al. 2016; Zhang, Burrows, et al. 2006; Setlow et al. 2016).

Fungi inactivation

Contamination with yeasts and molds is a serious problem in food industry which leads to enormous economic losses. However, the methods used so far i.a. high temperature, ozone treatment or application of high hydrostatic pressure have many drawbacks such as destruction of nutrients, environmental pollution or high operating costs. Inactivation of fungi with $scCO_2$ has not been extensively studied. However, comparison of the conditions necessary to inactivate *E. coli* and fungi indicates that fungi have a higher resistance to $scCO_2$. Garcia-Gonzalez et al. (2009) evidenced that in the same conditions, yeasts such as *Penicillium roqueforti*, *Aspergillus niger*, *Saccharomyces cerevisiae*, and *Candida albicans* are significantly less sensitive than most tested bacterial strains i.a. *Listeria monocytogenes*, *Salmonella typhimurium*, *Pseudomonas fluorescens*, or *Yersinia enterocolitica*. For yeasts, the reduction was equal or less than 2 while for mentioned bacteria it was in the range 3.5–5. However, it turned out that some bacterial strains as *Enterococcus faecalis* and *Alicyclobacillus acidoterrestris* show greater resistance (only to 0.3 log reduction) than yeasts (Garcia-Gonzalez et al. 2009). Qiu et al. explored the potential of scCO2 inactivation on molds of the species Aspergillus, Penicillium and Verticillium and the yeast *Debaryomyces hansenii* but they used peracetic acid as an additive which excludes this method from being used in the food industry (Qiu, Sun, and Connor 2011; Qiu et al. 2009). Lin et al. showed that ten minutes in supercritical conditions (6.89 MPa and 35 °C) is sufficient to inactivate wet yeast cells by 10^7-fold (Lin, Yang, and Chen 1992). Park, Choi, and Kim (2013) evidenced the efficiency of $scCO_2$ with water as co-solvent in elimination of *Penicillium oxalicum* spores from barley seeds. The optimal conditions obtained from Box-Behnken design and response surface methodology (10 MPa, 44 °C, 12 min and 231 μL H_2O for 3 g of barley seeds) resulted in a 6.8 log reduction of cfu. Similar results were obtained for wheat grains (Park et al. 2012). In both cases authors proved that the inactivation yield of *P. oxalicum* spores significantly increased with increasing amount of co-solvent (water) and was the most significant parameter for spore inactivation. However, further studies are needed to develop methods of fungi inactivation for wider range of food products.

Viruses inactivation

In addition to bacteria, food can be often contaminated with viruses. The most important from the point of view of food safety are noroviruses, hepatitis A and E and rotaviruses. However, to date, there are only few reports on the possibility of inactivating viruses with $scCO_2$ and they focus on stabilization of biological material for medical purposes. In these studies, peracetic acid or hydrogen peroxide were used as additives to $scCO_2$ (Qiu et al. 2009; Bernhardt et al. 2015; Perrut 2012; Efaq et al. 2014). Therefore, the developed methods will not be able to transfer into the food industry. So far, pure $scCO_2$ has only been used for inactivation of bacteriophage Qβ and ΦX174 in water. For pressure 0.7 MPa and an exposure time of 25 min, a greater than 3.3-log reduction in bacteriophage Qβ was achieved, while a nearly 3.0 log reduction was observed for phage ΦX174 (Vo et al. 2014). It was evidenced that CO_2 had better ani-virus activity than N_2O that suggests that process of viruses inactivation is related with acidification. Authors suggest that hydrogen ions that are products of carbon acid dissociation can penetrate protein coats of bacteriophages and dissolve the phospholipids (Vo et al. 2014).

Effect of pressure and temperature on the inactivation of vegetative cells

Many parameters affect the level of inactivation, the main ones are pressure, temperature, duration of treatment, depressurization rate, mixing, additives, type of medium, and level of contamination (Garcia-Gonzalez et al. 2007;

Perrut 2012; Ebara et al. 2004; Spilimbergo and Bertucco 2003). Examples of parameters which are efficient in bacterial inactivation are presented in Table 1.

In general, bacterial cells are quite resistant to high pressure due to their structure, so hydrostatic pressure above 100 MPa is necessary to their inactivation (Zhang, Burrows, et al. 2006). Pressures applied during $scCO_2$ inactivation are much lower which clearly indicates that this parameter is not alone factor responsible for microbial inactivation (Soares et al. 2019).

However, each microbial inactivation studies with $scCO_2$ reports the effect of pressure and temperature on the inactivation efficiency, because T and P are the main process parameters that control the properties of the supercritical fluid. The density of the $scCO_2$ decreases with increasing temperature generally. However, with the elevation of temperature the vapor pressure of the solute increases, so the solute became easily extractable. The solubility of CO_2 in media increases with the increasing pressure, but the highest-pressure dependency is observed in the vicinity of critical point (Wrona et al. 2017, 2019a, 2019b). These parameters also affect microbial inactivation to the greatest extent, directly affecting the characteristics the biological activity of microbial cells.

In general, inactivation of microorganisms accelerates with increasing pressure of CO_2. Accordingly, a shorter exposure time is required at higher pressures to inactivate the same level of microbial cells (Hong, Park, and Pyun 1997; Hong and Pyun 1999). Basically, the pressure controls both the rate of CO_2 solubilization and its complete solubility in the nutrient medium. Therefore, a higher pressure enhances the solubilization of CO_2, facilitating both acidification of the external environment when solubilizing compressed CO_2 in the liquid phase, and the interaction of CO_2 with cells. In addition, CO_2 at higher pressures exhibits a higher solvating ability, which also facilitates the elimination of vital components from cells and cell membranes.

Microbial inactivation is also sensitive to the applied temperature. The inactivation rate increases with increasing temperature. High temperatures stimulate the diffusion of CO_2, and can also increase the fluidity of the cell membrane to make penetration easier. Thus, increase in the temperature of the process stimulates an irreversible change in the cell membrane and, accordingly, the elimination of vital components from the cells. The decrease of the $scCO_2$ density with the temperature rising can be compensated by the increase of the pressure value (Lucien and Foster 1999; Razgonova et al. 2019).

The high value of the process temperature may cause decomposition of the thermolabile components existing in the plant material, which can provide deteriorate or spoil the quality of food in many cases (Spilimbergo, Elvassore, and Bertucco 2002; Duan and Sun 2003). On the other hand, studies on inactivation optimization of dietary supplements from *E. coli*, *S. cerevisiae* and *L. innocua* showed that temperature had a dominant effect. However, the effect of pressure was lesser (Fleury et al. 2018).

In addition to the above parameters, time is also a crucial factor in $scCO_2$ stabilization. During the process two or three phases can be distinguished. Usually the lag phase and exponential phase are distinguished. During the lag phase slow inactivation is observed which is associated with the penetration of CO_2 into microbial cells. During exponential phase this pool of CO_2 disrupts cell function (Garcia-Gonzalez et al. 2007; Kamihira, Taniguchi, and Kobayashi 1987; Hossain et al. 2015a, 2015b; Rao, Bi, et al. 2016). When CO_2 very quickly penetrates bacterial cells, lag phase is shortened so that only one-stage kinetics are observed (Garcia-Gonzalez et al. 2007; Zhang et al. 2007; Qiu, Sun, and Connor 2011). But always the duration of stabilization depends on the combination of pressure and temperature.

Since the effect of the above parameters on the properties of the solvent and the efficiency of inactivation is very complex, mathematical modeling and statistical tools are exploited for screening critical treatment factors. The main advantage of these methods is the screening of a large number of independent factors in a small number of experiments and the ability to evaluate the interaction between the different factors on the supercritical microbial inactivation (Silva et al. 2018). The most popular approach is to apply central composite design (CCD) or Box-Behnken design (BBD) that generate the best ratio between the number of factors and experiments performed to plan experiments and response surface methodology (RSM) to obtain polynomial quadratic Equation (5) and to determine optimal process parameters. Moreover, RSM allows for graphical presentation that depicts the effect of independent variables (factors) on the dependent variables.

$$y = \beta_0 + \sum_{i=1}^{n} \beta_{ii} x_i^2 + \sum_{i<j}^{n-1} \sum_{j=2}^{n} \beta_{ij} x_i x_j + e \qquad (5)$$

y – output variable, β_0, β_i, β_{ii}, β_{ij}, - coefficients of equation regression, $x_i x_j$ – input variables, e - error and residuals

Studies on apple juice inoculated with *Lactobacillus casei* (10^7 CFU/mL) showed that during the process at 15 MPa, 55 °C, 30 min and a 70% CO_2 volume ratio, the number of bacteria was reduced by more than 6 log cycles. Among the four tested factors, temperature, process time, and CO_2 ratio showed a positive effect on the log reduction of *L. casei*. However, the pressure value (10 and 20 MPa) had no significant effect on microbial inactivation (Silva et al. 2018). Authors revealed that among tested variables, CO_2 volume ratio has the most significant effect on the supercritical microbial inactivation although this parameter is often overlooked in research. This phenomenon can be explained by greater amount of supercritical carbon dioxide solubilized in apple juice which induces appropriately intensified changes described in "The mechanism of bactericidal action of carbon dioxide" section. However, optimization of $scCO_2$ process for microbial inactivation of dietary supplements inoculated with *S. cerevisiae*, *E. coli*, and *L. innocua* showed that temperature has a dominant effect whereas the impact of pressure is significantly limited (Fleury et al. 2018).

Mixing (agitation) is another factor that affects inactivation efficiency, but is often overlooked. In general, the more

Table 1. Summary of experimental $scCO_2$ conditions of microbial inactivation in food raw materials and products.

Type of bacteria	Material	Operational modes	P, MPa	T, °C	Time, h	Degree of inactivation, log	References
Lactobacillus casei	Apple juice	Batch	15	55	0.5	6	Silva et al. (2018)
Bacillus cereus	Growth media and	Continuous	20.5	60	4	8	Dillow et al. (1999)
Legionella dunnifii	biodegradable polymers			40	1.5	4	
Staphylococcus aureus,	(soya broth)			40	4	9	
Listeria innocua,				34	0.6	9	
Salmonella salford,				40	4	9	
Proteus vulgaris,				34	0.6	8	
Pseudomonas aeruginosa				40	4	8	
Escherichia coli				34	0.5	8	
Bacillus cereus	Baker's yeast	Batch	30	45	1	6	Ishikawa et al. (1997)
Bacillus subtilis				50	1	6	
Bacillus megaterium				40	0.5	2	
Bacillus polymyxa,				45	1	6	
Bacillus coagulans				40	0.5	2	
Saccharomyces cerevisiae	Physiological saline	Batch	20	35	2	7.5	Kumagai, Hata, and Nakamura (1997)
Escherichia coli						6.5	
Staphylococcus aureus,						5	
Aspergillus niger						5	
Escherichia coli	Growth media	Semi-continuous	15	35	1	8	Spilimbergo and Bertucco (2003); Smelt and Rijke (1992)
Lactobacillus brevis	Physiological saline	Semi-continuous	25	35	0.25	6	Spilimbergo and Bertucco (2003)
Staphylococcus aureus	BHIB	Batch	8	25	1	7	Erkmen (1997)
Escherichia coli	Nutrient broth	Semi-continuous	10	35	6	6–7	Spilimbergo and Bertucco (2003)
Salmonella typhimurium	Physiological saline	Semi-continuous	0.6	35	0.25	7	Spilimbergo and Bertucco (2003)
Staphylococcus aureus	Buffer solution	Batch	31	42.5	0.25	7	Sirisee, et al. (1998)
Escherichia coli						7	
Bacillus subtilis	Physiological saline	Semi-continuous	7.4	38	2.5 min	7	Spilimbergo, Elvassore, and
Pseudomonas aeruginosa						7	Bertucco (2002)
Escherichia coli	*Coriandrum sativum* and *Rosmarinus officinalis*	Batch	8	40	0.75	5	González-Alonso et al. (2020)
Escherichia coli	LB broth, apple and orange juice	Continuous	36	36	0.25	8	Ortuño et al. (2012)
Staphylococcus aureus	Chicken, turkey ham	Continuous	35	36	0.33	7–8	Benedito et al. (2015)
Escherichia coli	and dry-cured pork				2.25	7–8	
Alicyclobacillus acidoterrestris	Apple	Batch	60	50	0.3	1	Porębska et al. (2017)
				75	0.6	3.5	
Staphylococcus aureus	Raw salmon	Continuous	22.5	33	2	5.3	Cuppini et al. (2017)
Pseudomonas fluorescens	Raw milk	Batch	20,7	35	10 min	5.02	Werner and Hotchkiss (2006)
Listeria monocytogenes	Orange juice	Batch	6.18	35	3	7	Wei et al. (1991); Rawson et al. (2012)
Lactobacillus plantarum	Coconut water	Batch	6.8	30	6 min	8	Valley and Rettger (1927)
Mesophilic bacteria	Pork raw meat	Batch	6	25	1	5	Cappelletti, Ferrentino, and Spilimbergo (2015)
Total viable count	Ground pork	Batch	13.8	35	2	2	Huang et al. (2017)
Mesophilic bacteria	Coriander leaves	Semi-continuous	10	50	2.5	4	Zambon et al. (2018)
Bacterial reduction	Tempeh	Continuous	7.6	31	20 min	2.4	Kustyawati et al. (2018)
Mold reduction						6.5	
Aerobic bacteria	Coconut water	Continuous	34	25	6 min	5.6	Damar, Balaban, and Sims (2009).

dynamic the mixing during the process, the greater the reduction in the amount of microorganisms, which is associated with the greater amount of CO_2 in the food subjected to inactivation. Without mixing, only cells present in the surface area of food are directly affected by $scCO_2$, while deactivation of the remaining cells depends on diffusion through the media (Boannaillie and Tomasula 2015).

Currently, the different methods to enhance the effect of $scCO_2$ have been proposed such as the application of successive cycles of pressurization and depressurization or use of additives (Rao, Wang, et al. 2016; Chen, Temelli, and Gänzle 2017; Silva et al. 2013). Studies on inactivation of fungi indicate that application of H_2O as co-solvent also can significantly improve the efficiency of $scCO_2$ (Park, Choi, and Kim 2013; Park et al. 2013). Other additives commonly used to sterilize medical products such as hydrogen peroxide, ethanol, acetic anhydride, peracetic acid, or a mixture of them, although are very effective, cannot be used in the food industry.

To improve the efficiency of supercritical carbon dioxide, combinations of different techniques are also introduced. Acoustic cavitation generated by ultrasound is effective

against live microorganisms and this technique is most often combined with $scCO_2$. Ultrasounds provokes alternating low- and high-pressure waves of liquids which leads to the formation and collapse of small vacuum bubbles that are responsible for damages caused to cell walls and membranes of bacteria or yeasts leading to their inactivation (Koubaa, Mhemdi, and Fages 2018; Ortuño, Balaban, et al. 2014; Ortuño, Quiles, et al. 2014; Ferrentino, Komes, and Spilimbergo 2015; Sara, Martina, and Giovanna 2014). The $scCO_2$ combined with ultrasound is effective in inactivation of *E. coli* and *S. cerevisiae* in liquid and solid matrices in much shorter time (1–2 min) compared to $scCO_2$ alone (25 and 140 min, respectively). TEM images showed that cells treated with $scCO_2$ and ultrasound had drastically reduced cytoplasm and many empty regions inside. The *E. coli* cell wall was totally disrupted. However, the cell wall of *S. cerevisiae* lost their layered structure and in some regions was not continuous. It seems that activity of $scCO_2$ is enhanced by the cavitation phenomenon which leads to rupture the bacterial wall and membrane as well disintegration of cell content and finally accelerates the leakage of vital constituents and leads to microbial cells death (Ortuño, Quiles, et al. 2014). The effectiveness of this method against *Listeria monocytogenes* and *Salmonella spp*. in dry cured ham and fresh cut coco-nut, two species important from food industry point of view has also been confirmed (Ferrentino, Komes, and Spilimbergo 2015; Sara, Martina, and Giovanna 2014).

The effectiveness of $scCO_2$ also depends largely on the type of food, including whether it is liquid or solid. The experiments by Sirisee, Hsieh, and Huff (1998) showed that significantly longer treatment time is necessary for inactivation of *E. coli* and *S. aureus* in ground beef sample compared to phosphate buffer. Lower efficiency in solid food is associated with lower water content and successively reduced amount of CO_2 dissolved in food matrix as well as with the protective effect of other food ingredients such as proteins and fats. On the other hand, $scCO_2$ induces changes in properties of solid food. Visual observations revealed that color of minced meat after process changed to color of cooked minced meat. One reason can be interaction of CO_2 with myoglobin which causes the formation of metmyoglobin and gives the dark color. It is also evidenced that the pressure and temperature applied during $scCO_2$ process lead to protein denaturation. Studies by Messens et al. (1997) showed a denaturation of such sarcoplasmic protein in meat as phosphorylase b, creatine kinase, triosephosphate isomerase and one unknown protein, which covers the red color of the sarcoplasm. $scCO_2$ is an effective technique for the microbial inactivation of vegetables and fruits. However, changes in the structure and color of biologically active compounds that occur during the process make their industry application limited.

Commercialization and industrial applications

Several patented systems and industrial equipment (also for continuous processes) have been available for over a decade from manufacturers such as the Praxair or GEA groups. However, industrial applications are still under development and has not been implemented to the food industry on commercial scale. Until now only NovaSterilis offers systems for microbial inactivation of biomaterials as allograft tissues, including skin, ligament, tendon, and bone as well as therapeutics. For food microbiological stabilization potentially, one of the three existing industry plant of SFE process, that can be used for batch inactivation, is placed in Łukasiewicz - New Chemical Syntheses Institute in Puławy, in Poland. Plant can work under pressure up to 530 bar (53 MPa) and temperature up to 100–120 °C (373.15–393.15 K) providing ECO and HACCP certificates.

The main problem is the cost of $scCO_2$ in comparison to thermal pasteurization. For comparison, the cost of the process in plant placed in Puławy is approximately a 5 euro per 1 kg of the feedstock and is about 10 times more expensive than a thermal process (Wrona et al. 2019b). It is worth noting that high pressure is especially suitable for products with low protein content as apple and grapefruit juice, beer, sake or solid fresh cut vegetables. Due to modifications of proteins under high pressure which results in texture modification, for products with high protein content, the process with uses CO_2 microbubbles at pressure lower than those required for $scCO_2$ is more useful (Ribeiro et al. 2019; Picart-Palmade et al. 2019).

Most studies on the effectiveness of the sc-CO_2 process have been conducted in batch systems. However, a development of continuous process is highly desirable from industry point of view. Until now, the continuous pilot system has been tested quite well in the production of orange juice. Although it was efficient in reduction of natural flora of juice (i.a. molds, yeasts, and bacteria), obtained results also revealed that the process carried out cannot replace pasteurization for long storage at room temperature but is a new mild technology for the stabilization of orange juice that retains its physicochemical, antioxidant, and sensory features (Fabroni et al. 2010). Low efficiency in continuous mode is due to the short scCO2 operation times, because time is one of the main parameters affecting the degree of microorganisms' deactivation. Therefore, pseudo-continuous systems in which at least two reactors can work in parallel and allows longer processing time and better CO_2 diffusion should be more effective. Another solution improving the inactivation efficiency is a combination of scCO2 and high-power ultrasound. Studies on pineapple juice confirmed that combination of these two techniques completely inactivated natural microbial in a continuous flow system and during storage microorganisms were not able to recover. The method used also allowed to preserve quality of the product, for example vitamin C levels were only 8% lower (Paniagua-Martínez et al. 2018).

Supercritical fluid due to its physicochemical properties could play a double role in the food industry. In addition to microbial inactivation, in the same process $scCO_2$ can extract a wide range of agents extremely harmful to human health such as pesticides, dioxins, mycotoxins and polychlorinated biphenyls. Therefore, the microbial inactivation

by supercritical CO_2 can have a positive effect on food quality by reducing the level of toxic compounds. So far it was evidenced the effectiveness of this method for isolation of pesticides such as organophosphates, organonitrogenates, carbamates, pyrethroids, and imidazole from various food matrices (orange, sweet potatoes, green beans, honey, banana flour) (Rissato et al. 2004; Sartori et al. 2017; Lehotay and Valverde-Garcia 1997; Yousefi et al. 2020). These analyses mainly relate to the analytical approach and are developed to determine the level of toxic compounds in food. However, the effectiveness of $scCO_2$ in extraction of mentioned substances should be transfer to higher industrial scale. Further studies that could correlate the effectiveness of microbial inactivation together with elimination of harmful substances are necessary and can be a new interesting trend for food industry.

Another aspect can be changes introduced to structure of the food during process of food inactivation. Studies by Matsubara et al. (2021) presented the possibility of using carbon dioxide in a supercritical state to enhance the resistant starch level in brown rice. Obtained product has health-promoting properties because higher resistant starch content contributes significantly to blood glucose level maintenance in cooperation with dietary fiber.

Conclusions

Since pasteurization, sterilization, and inactivation of microorganisms are of primary importance in the food, pharmaceutical, and biomedical industries due to the improvement of the quality of the final product and its safety, treatment with $scCO_2$ is extremely desirable because it avoids excessive heat treatment or radiation treatment that does not can always be used, especially with regard to food.

As discussed in this article, over the past two decades, considerable fundamental work has been done to describe the interactions between $scCO_2$ and microorganisms in both the vegetative state and in spores in order to better investigate the effect of stabilization by subcritical and $scCO_2$ in combination with modifiers and effectively control the processes of non-thermal inactivation.

Scientific teams working in the field of food sciences are developing new technologies to eliminate pathogenic factors in food and spoilage of food products by microorganisms, and the results of experiments suggest achieving a positive result in these aspirations. In the past decade, technologies have been developed for the preservation and conservation of food products by means of $scCO_2$ treatment, and considerable success has been achieved in this field. So far, the best results have been obtained with matrices with a high water content. It was proved that $scCO_2$ significantly reduces *Escherichia coli* in apple and orange juice (36 MPa, 36 °C, 15 min) and *Listeria monocytogenes* in orange juice (6.18 MPa, 35 °C, 3 min) (Ortuño et al. 2012; Wei et al. 1991; Rawson et al. 2012). Slightly lower effectiveness was noted for *Staphylococcus aureus* in raw salmon (22.5 MPa, 33 °C, 2 min) and *Pseudomonas fluorescens* in raw milk (20.7 MPa, 35 °C, 10 min) (Cuppini et al. 2017; Werner and Hotchkiss 2006).

$ScCO_2$ treatment should not only improve the quality of food, but also increase the shelf life and (long-term) safety by inactivating pathogens in order to replace other food preservation methods in the future. Therefore, further research is needed to explain the effect of $scCO_2$ on food processing in relation to shelf life and food safety. In addition, it is important that the effect of $scCO_2$ treatment on the organoleptic properties and nutritional value of both liquid and solid foods is more thoroughly studied.

Finally, it should be noted that since microbial inactivation with $scCO_2$ has great potential for improving food safety and quality, many technological and regulatory barriers need to be improved (e.g., further process optimization, industrial scale development, acquisition of the most complete data on organoleptic properties and timing storage, quality certification, etc.), which is still necessary before new technologies can get these benefits. More research is still needed on efficiency of $scCO_2$ in real samples. So far most of the tests are carried out on materials contaminated with specific bacterial strains, not taking into account matrix effect that hinders fluid access to bacterial cells or matrix compounds. Some analysis indicate that microbial inactivation of real samples is not so efficient as inactivation of spiked samples. Another aspect is the calculation of cost-effectiveness of the processes presented in the literature. To reach commercial level and acceptance of this "green" technology above mentioned issues should be clarified.

Author contributions

Literature survey and first draft writing were done by O.W., B.B., M.P.R., A.M.Z., T.K.K., W.P., K.S.G., K.R. and final draft including the revisions were accomplished by B.B. and K.R. All authors have read and agreed to the published version of the manuscript.

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