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Innovative strategies and nutritional perspectives for fortifying pumpkin tissue and other vegetable matrices with iron

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ABSTRACT

The present review article focuses on different technological strategies and nutritional perspectives having added advantage to human health in fortifying vegetables matrices with iron. An introduction to the main aspects related to iron deficiency consequences is resumed in order to understand the importance of developing new strategies for improving iron intake. In this sense, the tendencies and alternatives will also be discussed. Emerging technologies like impregnation or dry infusion are presented as sustainable options for obtaining structured fortified vegetables. A review about the usefulness of edible covers for stabilizing micronutrients and/or probiotic microorganisms in fortified matrices is also analysed. Since iron deficiency still continue to be a worldwide health issue, innovating in food fortification remains a challenge for researchers and food manufactures.

1. Iron deficiency and functional health consequences

Iron has several vital functions in the body, such as a carrier of oxygen to the tissues from the lungs by red blood cell haemoglobin, as a transport medium for redox systems into cells, as well as participating in enzyme arrangements. Iron deficiency (ID) is the result from reduction of iron stores; and happens when iron absorption cannot respond to metabolic demands for instance, to sustain growth and to replenish iron loss [1]. Low intake of bioavailable iron and the increase of the mineral requirements due to growth, pregnancy, and blood loss caused by pathogenic infections, are the primary causes of ID [2,3]. The more severe stages of ID are associated with anaemia, that occurs when there is an inadequate amount of red blood cells because the falling down haemoglobin concentration (<110 g/L) as a consequence of lack of iron [1]. It is important to highlight that there are mild-to-moderate forms of ID in which, although anaemia is not diagnosed, tissues are functionally impaired [4,5]. Haemoglobin concentration is often used as indicator of anaemia, nevertheless its measurement alone do not determine the cause of anaemia and when is used with other indicators of iron status, it can provide information about the severity of ID [2,4]. Anaemia may result from a number of causes, being approximately 50% of cases due to ID [2].

On the one hand, anaemia is considered worldwide as a public health problem, affecting over 30% of the world's population - two billion people – with important consequences on human health, social and economic growth. It affects all stages of the cycle life but has a higher incidence rate in pregnant children (43%), women (38%) and women in reproductive age (29%) [2]. The functional health consequences of ID are several, including cognitive performance, behaviour and physical growth of infants, the immune status and morbidity from infections, and the physical capacity and work performance of adolescents and adults. Meanwhile, ID anaemia during pregnancy, enlarges perinatal risks for mothers, neonates and overall infant mortality [3].

On the other hand, there is a concern about the increase in consumption of fortified foods since high iron stores may compromise cardiovascular diseases, type 2 diabetes and cancer [6]. By the moment, there is scarce scientific report which relates iron intake to chronic disease and this concern has not been completely resolved. Nevertheless, benefits due to prevention of ID have outweighed the associated potential risks, in both developed and developing countries [6].

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ID can be prevented or treated by the iron-fortified foods; which are considered so far the most cost-effective strategy to provide additional iron to populations with a high prevalence of anaemia [2,6].

Most countries have adopted mass fortification as public health policy, and hence, the food vehicles selected have been widely consumed by the target groups. It depends exclusively on the availability and accessibility of the population. Among the most common we can mention the salt, sugar, flours, cereals and dairy products. It is noteworthy that minimal processed fruits and vegetables have not been included for mass fortification or enriching programs with iron.

Recently, the scientific community has expressed its concern that, despite the efforts made in many countries in the last decades, anaemia is still holding its prevalence in their populations. In concordance, the World Health Organization has included the anaemia as the second global goal of nutrition by the year 2025, aiming to reduce anaemia in women of reproductive age by 50% [2].

From a nutritional perspective, food fortification also implies the appropriate selection of iron compound (water-soluble iron are considered to be the most bioavailable), and the presence of enhancers added during the food formulation or naturally present in the matrix food such as Vitamin C, Vitamin A, amino acids, organic acids and probiotics, are some of the strategies to ensure bioaccessibility and bioavailability of the mineral, and the efficacy in improving iron status in individuals [7]. Before becoming bioavailable, bioactive compounds must be released from the food matrix during the pass through the gastrointestinal tract [8]. Thus, bioavailability includes the term bioaccessibility, and both terms are part of nutritional effectiveness, as not all the amounts of bioactive compounds are used effectively by the organism [9]. On the other hand, there are other extrinsic factors to consider at the moment of iron-food fortification, *e.g.* the iron status of the target population, and some compounds from diet that can interact with the mineral and, consequently, reduce the bioaccessibility and bioavailability.

2. Alternatives for improving iron intake

Iron in food is present in two forms: haem iron and non-haem iron. Iron is absorbed mainly in the duodenum by different mechanisms according these forms. Haem iron is only provided by animal food, and this iron form is almost unaffected by dietary factors, with an average absorption from meat-containing meals of around 25%, ranging from about 40% during iron deficiency to about 10% when iron stores are replete [1].

Non-haem iron is present in plant-based foods in the ferric form (Fe^{3+}), which has low solubility. The ferric iron is degraded during digestion in the gastrointestinal tract owing to the action of enzymes and hydrochloric acid, presenting a low average absorption (2%–10%), depending on the balance between inhibitors and enhancers of iron absorption present in the composition of the meal; which represents a key factor in the diet of people without access to animal foods. In general, enhancers are dietary components that can reduce ferric iron to ferrous iron (Fe^{2+}), such as ascorbic acid, carotenes, organic acids and some amino acids. By contrast, major inhibitors of iron absorption include tannins, phytic acid, polyphenols, calcium, and casein [6,10].

Bio-fortification is the process for increasing the content and bioavailability of essential vitamins and minerals in staple crops, through plant breeding or agronomic practices, to improve nutritional value of crops [11,12]. Genetic modification of plants in order to obtain a final plant food with a higher iron content, is another approach for which there are high expectations and may be more cost effective in the long run [13–15]. Agronomic bio-fortification provides an immediate and adequate route to enhance micronutrient concentrations in edible crop products. Nevertheless, the effectiveness largely depends on the bioavailability of micronutrients throughout all the way from the ground to plants, trespassing the food processing and ending in the human body. For instance, de Valença et al. [16], reported that agronomic biofortification can be useful in rising yields and nutritional quality for certain crop-micronutrient combinations; especially zinc and selenium on wheat and maize, whereas iron has shown little potential to date. On the other hand, supplementation and diet diversification programmes work best in centralized urban areas, while agronomic biofortification is the best approach to reach rural populations [12].

Food fortification and supplementation are the most commonly used strategies to alleviate micronutrient deficiencies among humans [16,17].

Target fortification and/or market-driven fortification seem to be a practical option in the improvement of public health or segments of population, for instance in women and children with iron deficiencies. From a food technological point of view, iron is considered the most difficult micronutrient to fortify foods due to the reactivity of the mineral with other components of the food matrix, altering the product acceptance and shelf life. Among others, metallic aftertaste, rancidity produced by fat oxidation, undesirable colour changes, textural properties changes, and vitamin degradation, are some of the problems that were not completely solved [6,18–20].

Commonly available fortified products include non-structured and formulated foods. Some examples are the fortifying of wheat flour and other cereal products, including breakfast cereals with electrolytic iron; dried infant foods and milk powder with ferrous sulphate or ferrous gluconate, plus ascorbic acid; chocolate drink powders, cereal-based complementary foods and salt with ferric orthophosphate or ferric pyrophosphate [6]. Fish sauce, soy sauce, curry powder, green leafy vegetable sauces could be adequate matrices to mix with iron source such as ferric sodium ethylenediaminetetraacetate (NaFeEDTA), ferrous sulphate, and ferric pyrophosphate [10,21]. More recently, extruded rice or bouillon cubes broth fortified with ferric pyrophosphate were also studied [22]. In contrast, impregnation or vacuum impregnation allows the introduction of bio-active compounds into food matrix without destroying vegetal structure, but inducing changes in their behaviour during further processing [23].

3. Impregnation technology

The concept of "hurdle technology" is based on the combination of barriers to assure microbiological stability and food safety, being temperature, pH and water activity (a_w) reduction, examples of the main barriers adopted. This technology permits to develop preserved fruits like peaches, mango, papaya, pineapple [24]. In this process, calcium addition, improves fruit texture by interacting with pectin chains and therefore avoiding their solubility [25]. Soon, this technology was turned into a tool for introducing biological active compounds inside vegetal matrices. Impregnation with vitamins and/or calcium and zinc were the first to be studied and had been widely reported [26–29]. With regards to iron, the effect of its incorporation on the kinetics of osmotic dehydration as well as, on other characteristics of apple tissue, were studied [30,31]. Apple slices (var. *Granny Smith*) were fortified through a vacuum impregnation process by Barrera et al [30]. Taking into account the Reference Daily Intake (RDI) established for iron (18 mg/day); the authors stated that 200 g of the vacuum impregnated samples supplied 43.57% of the RDI. They also reported that when an osmotic dehydration process was applied after vacuum impregnation; iron loss to the medium occurred. In that case, 200 g of the samples

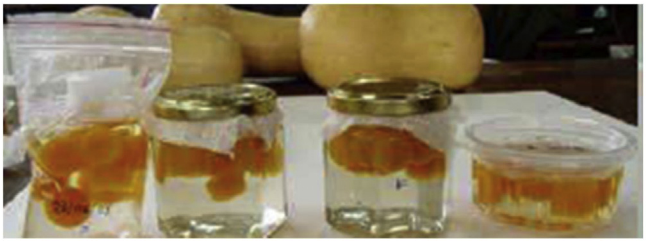

Fig. 1. Picture of pumpkin cylinders impregnated and fortified with iron and ascorbic acid.

vacuum impregnated and dehydrated by a subsequent osmotic process, supplied 35.44% of the RDI.

Vacuum impregnation and/or ultrasound were applied in order to improve the incorporation of iron into vegetable tissues. In this sense, Hironaka et al. [32] could fortify whole potato tubers with ferric pyrophosphate solution. Vacuum pressure of 1000 Pa followed by atmospheric pressure restoration allows providing 6.4 times higher iron content compared to raw potatoes. More recently, Mashkour et al. [33] demonstrated that vacuum impregnation with an ultrasound pre-treatment, significantly enhanced the iron content in fortified-cooked potatoes. Miano et al. [34] took advantage of the hydration process assisted by ultrasound to introduce iron in carioca beans. They demonstrated that ferrous sulphate could be incorporated during the hydration process, describing similar kinetics behaviour to the water uptake. In addition, ultrasound accelerated this process, achieving 60.1 mg iron/100 g w.b., in contrast to 34.4 mg/100 g. when the beans were hydrated without ultrasound. However, the inconvenient of using iron in solutions is mainly the possible changes in colour, flavour or interactions with other compounds of food. The colour of iron compounds is often a critical factor when fortifying lightly coloured foods [34]. Alternatively, ultrasound pre-treatment, applied for improving drying, could be profited to incorporate compounds of interest (nutrients) into the food matrix, enhancing the nutritional quality of dry products [35,36]. Rojas et al. [35] dehydrated pumpkin cubes by means of an ethanol suspension of ferrous sulphate microparticles. They registered iron content of 24 mg/100 g in dehydrated fortified pumpkin; and iron content increased up to 3 mg/100 g at 25% w.b, when a pre-treatment with ultrasound was applied.

Special attention was paid by the authors for pumpkin mesocarp (*Cucurbita moschata* Duchesne ex Poiret) since this kind of pumpkin does not contain neither lignin nor phenolic compounds that can inhibit iron absorption, and in addition it is a good source of carotenoids compounds which can increment iron bioavailability [37]. Moreover, pumpkin *Cucurbita moschata* is one of the most consumed vegetables in Argentina and an expanding interest in this vegetable has also been reported in other countries [35]. The good performance of pumpkin tissue for supporting iron could be demonstrated by de Escalada Pla et al. [37]. Fig. 1 shows a picture of pumpkin pieces immerged in hypertonic osmotic covering solution. This impregnated solution was formulated with glucose and sucrose, as a_w depressors; citric acid for pH reduction; *L*-(+)-ascorbic acid as a hydrogen donor, in synergism with iron as well as a scavenger for free radicals to preserve carotenes from degradation; potassium sorbate was added for inhibiting yeast and mould growth; finally, vanillin was used as a flavouring agent. In that opportunity, iron was introduced in the vegetal matrix during the cooling step after blanching and before the final immersion in hypertonic solution. The cooling solution contained 500 ppm iron and 500 ppm of ascorbic acid.

Pumpkin is a good source of carotenoids compounds but not of iron. With the process applied a final product with a_w value of 0.930 was obtained and (11 ± 5) mg/100 g of iron could be incorporated. The product was stored for 60 days at 25 °C, being the ascorbic acid loss, the factor that stablished the shelf-life of 23 days in the condition assayed [37]. By contrast, the use of high ratio of solution:pumpkin in cooling solution as well as hypertonic solution represented an operative matter to be solved.

Among the technologies commonly used to reduce water content in vegetable food matrices, dry infusion could be considered a good alternative from a sustainability point of view, where water consumption is drastically reduced and water partially drained from the tissues demands low energy consumption [38]. A dry infusion process was proposed as more sustainable alternative for introducing iron in pumpkin tissue [39]. Briefly, the methodology consisted of sprinkling pumpkin pieces with the dry powdered ingredients. The water for dissolving the ingredients came from the same vegetal tissue which began to flow from the pumpkin cylinder to the surrounding concentrated in soluble solids. No extra drinking water must be added. Furthermore, a subsequent air drying was applied in that opportunity, in order to obtain a_w values lower than 0.90. A detailed study of the effect of iron and ascorbic acid addition on dry infusion process and final colour of pumpkin tissue can be deepen in Genevois et al. [39]. Pumpkin pieces were disintegrated in order to homogenate the sample for subsequent determination. In Fig. 2 it can be observed a picture of the product obtained and disintegrated through the different formulations tested. A control system, without fortification, was also included in the picture for comparative purposes. It could be noted that system 6 showed the smallest colour alterations due to the fortification and process applied. Authors stated that the addition of iron or ascorbic acid significant reduced L^* value and Chroma (Chr), being this latter the most significant parameter affected by the drying process. It could be concluded that the colour difference observed was mainly related to chromatic coordinates: a^* and b^* value changes, and in part associated to ascorbic acid destruction due to the non-enzymatic browning reaction chain [40,41] and carotenoids compounds degradation [42]. Therefore, once the dry infusion processes were optimized, the main challenge was to find the way of reducing the rate of the reactions responsible for ascorbic acid destructions and carotenoids lost. In this sense, a preliminary assay using edible tapioca starch coating was tested and a protective effect in terms of the colour of pumpkin cylinders during drying, could be registered [39]. It must be highlighted that when drying infusion was carried out, the incorporation of iron into pumpkin tissue was quadruplicated compared to impregnation with hypertonic solution [43]. Nevertheless, stability of ascorbic acid as well as carotenoids compounds had to be improved in order to ensure sensory as well as nutritional purposes. The Table 1 summarized different options for incorporating iron to structured vegetable tissues while on the next section, the use of edible coating for nutritional and sensory stability will be discussed. As can be observed in Table 1, the iron content of these vegetable fortified matrices was similar or superior to the iron quantity reported in foods considered sources of iron. For example, the liver of goose, pork and chicken presents an iron content of 23–10 mg/100 g, sesame flour or seeds 15.17 mg/100 g, cooked spinach 3.5 mg/100 g, and soybeans seeds 15.7 mg/100 g [44].

4. Edible coating technology as strategy for improving nutritional and sensory properties

4.1. Edible film and coating technology

Edible films and coatings may be defined as continuous and thin layers of edible material, formed on or placed between food components [23,45,46]. They can be used to improve appearance or texture, or to reduce surface transport phenomena; mainly water

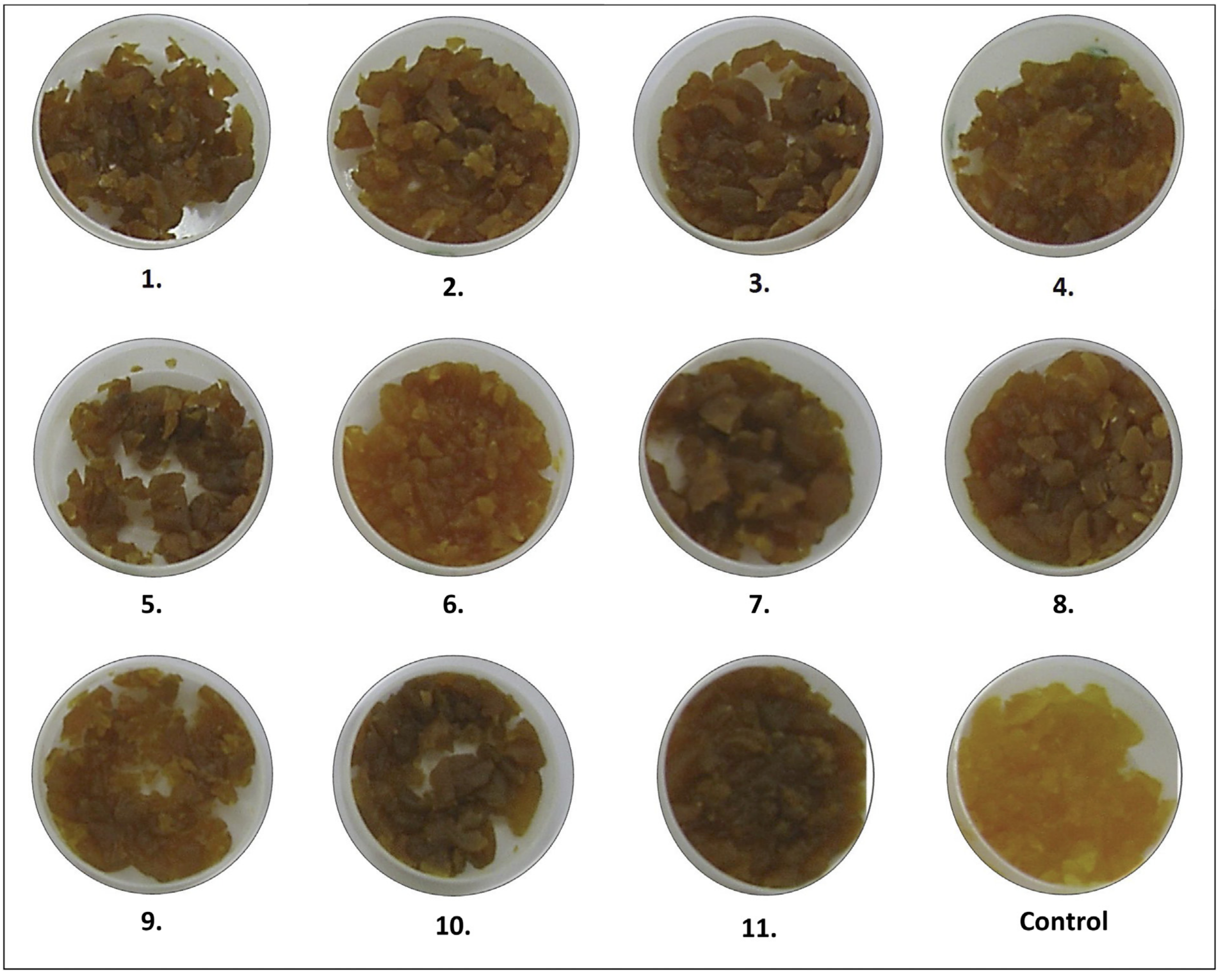

Fig. 2. Pumpkin fortified with iron and ascorbic acid by dry infusion, after air drying.
Numbers corresponds to systems from central composite design. Control system (C), without iron fortification, is also included [39].

losses or gains from food. Edible films and coatings can also be used as a carrier of additives in order to contribute to the stability of the product [47].

Among the emerging technologies for optimizing the food preservation, the use of edible films or covers, that can impart specific functional properties, emerges as a novel alternative.

In the case of edible coatings, the technique consists of applying a thin layer of an edible material on the surface of a food in the form of a membrane to reduce the loss of moisture and the migration of solutes, the gas exchange, the respiration and the rate of oxidative reaction, prolonging the food shelf life (Fig. 3) [48,49].

The benefits of the edible coating application are associated with the control of moisture migration and the transport of gases (O_2, CO_2, ethylene); the restriction of the exchange of volatile compounds between the product and the environment, preventing the loss of volatile aromatic compounds and colour components. Additionally, the migration of oils and fats and the transport of solutes can be also delayed. Finally, edible coating might improve mechanical properties against handling and impart additional structural integrity to food.

The structural components of edible coating are classified into three categories: lipids, hydrocolloids and mixtures [47]. Hydrocol-

Table 1
Vegetables matrix fortified with iron compounds applying different technologies.

Vegetable Matrix	Iron Salt	Iron content[a]	Technology	Reference
Potato tubers (Toyoshiro)	Ferric Pyrophosphate	4.1	Vacuum impregnation	[32]
Potato tubers (Snowden)	Ferric Pyrophosphate	4.4	Vacuum impregnation	[32]
Apple slices (var. *Granny Smith*)	Ferrous gluconate	3.2	Vacuum impregnation + Osmotic dehydration	[30]
Apple slices (var. *Granny Smith*)	Ferrous gluconate	3.9	Vacuum impregnation	[30]
Carioca kidney beans (*Phaseolus vulgaris*)	Ferrous Sulphate	34.4	Soaking hydrating process	[34]
Carioca kidney beans (*Phaseolus vulgaris*)	Ferrous Sulphate	60.1	Soaking hydrating process + Ultrasound	[34]
Pumpkin (*Cucurbita moschata*)	Ferrous Sulphate	11	Impregnation + Osmotic dehydration	[37]
Pumpkin (*Cucurbita moschata*)	Ferrous Sulphate	44	Dry Impregnation	[39]
Pumpkin (*Cucurbita moschata*)	Ferrous Sulphate microparticles	24	Ethanol drying	[35]
Pumpkin (*Cucurbita moschata*)	Ferrous Sulphate microparticles	33	Ethanol drying + Ultrasound	[35]
Pumpkin (*Cucurbita moschata*)	Ferrous Sulphate	54	Dry Impregnation + Tapioca starch coating containing iron	[23]
Pumpkin (*Cucurbita moschata*)	Ferrous Sulphate	35	Dry Impregnation + HPMC coating containing *L. casei*	[45]

[a] Iron content mg/100 g.

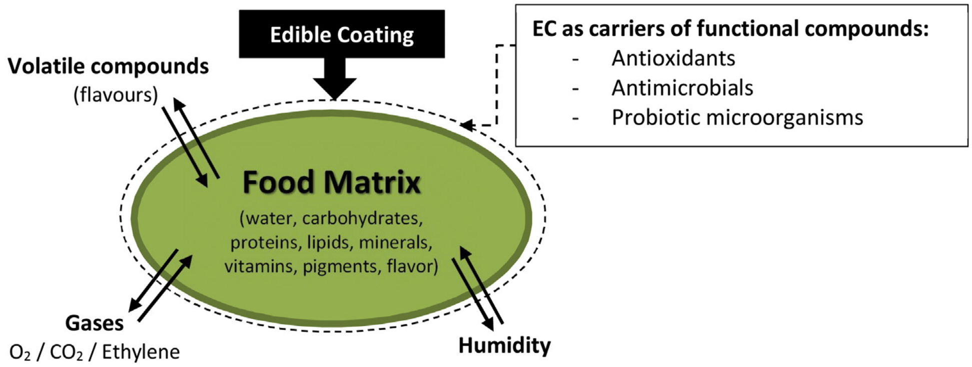

Fig. 3. Functional properties of the edible covers/coatings in foods.

loids include proteins and polysaccharides, while blends consist of a combination of polysaccharides, proteins, and/or lipids. It should be noted that some of the properties of the edible coating, such as the cohesiveness or structural characteristics of the film-forming matrix, will depend on the type of hydrocolloid or biopolymer used, the formulation (presence of plasticizers and/or additives), the rate of gelation, drying rate, *etc.* [50].

4.2. Advantages of edible coating application in fortified food with iron

Fortification with iron may present some challenges that have driven efforts to develop alternative technologies in order to address certain problems. One of them is the influence of the iron presence on the organoleptic properties of fortified foods, *i.e.* undesirable changes in colour, taste or texture. Iron catalyses oxidative processes in fatty acids, vitamins and amino acids, and consequently alters sensory characteristics and decreases the nutritional value of the food [51]. Some researchers have studied the application of edible coating to counteract these effects. Coatings can help to reduce unwanted organoleptic changes or act as support for micronutrients such as iron, in order to minimize interactions with other nutrients in the food. Simultaneously, the nutritional value of coated fortified food is improved. The edible coating promotes the supply of vitamins and minerals mainly by avoiding its interaction with other food components as the inhibitory compounds. Therefore, the encapsulation of bioactive in edible coatings improve the functionality and enhance the nutritional value of foods [52].

Edible coatings were applied on iron-fortified rice premix (IFRP) by soaking and spraying method. Hydroxypropyl methyl cellulose (HPMC), methyl cellulose (MC), combination of HPMC and MC, zein, palmitic acid and stearic acid were tested as coating matrices. Iron content ranged from 1.33 mg/g to 7.11 mg/g and 1.61 mg/g to 4.49 mg/g, respectively. Steam process reduced iron content. After washing twice with distilled water, retention of iron in these coated IFRP ranged from 87.34% to 89.39% ($P > 0.05$) as compared to 39.12% in uncoated IFRP. The authors concluded that the coating of iron-fortified rice premix with different coating material is effective in reducing the leaching losses of iron in washing water [53].

Genevois et al. [23] produced a refrigerated ready-to-eat food fortified with iron and ascorbic acid using pumpkin (*Cucurbita moschata* Duchesne exPoiret) and applied a dry infusion as well as coating technology. It was observed that the presence of both iron and ascorbic acid in the vegetable matrix (control system) produced the browning of the product during storage. The edible coatings application based on carrageenan or tapioca starch was proposed in order to improve the product stability. The ascorbic acid degradation in the tissue of pumpkin was significantly reduced when iron was supported in a starch-based coating. The result of an *in vitro* gastric and intestinal digestion assay indicated that when iron was in the coating, its solubility at pH 2 was lower than control (pumpkin fortified with iron and ascorbic acid in the tissue) and tended to improve at pH 8. Consequently, this was interpreted as a better accessibility of iron at intestinal lumen level avoiding gastric side effects, which represent an additional nutritional advantage.

DeŃobili et al. [54] studied the possibility of formulating ascorbic acid coating for an iron fortified product based on *Cucurbita moschata* Duchesne ex Poiret by dry infusion. Edibles coatings of alginate were proposed and different strategies were applied. In this case, iron was impregnated in the tissue while ascorbic acid was supported in the coating. The food colour stabilization was also studied at different process and storage conditions. Alginic acid is a natural polysaccharide harvested from brown algae which has film-making capacity in presence of divalent cations such as calcium, strontium, iron, magnesium [55]. Ascorbic acid is the most potent enhancer of iron absorption, both in its natural form in fruits and vegetables and when added as a free compound [56]. It has also been shown that carotene improves *in vitro* iron absorption and in human absorption studies, by a mechanism that could be associated with its provitamin-A activity [57]. Previous studies have reported that compartmentalizing ascorbic acid in a network of edible film helps achieve stabilization and at the same time, prevents ascorbic acid interactions with oxygen and other preservatives, nutrients or food components. Besides, the films may provide antioxidant activity localized at the interfaces [40,58,59]. It was demonstrated that ascorbic acid initial content was enhanced when it was incorporated in the coating, mainly due to the avoiding of ascorbic acid reactions with matrix components. Additionally, a_w reduction of the product at a level of 0.76 or the low storage temperature (18 °C) helped slowing down the ascorbic acid loss rate. The study of the colour changes revealed that all samples suffered slight darkening during storage. Faster b^* value and Chr reduction rates were observed for systems covered with a coating supporting ascorbic acid. It was attributed to the development of browning reactions into pumpkin matrix, without the assistance of ascorbic acid to prevent iron interactions with vegetal components and carotenes degradation. However, in general, the mentioned colour changes occurred at a slower rate than ascorbic acid loss. According with these results, it was concluded that it was possible to stabilize ascorbic acid in an alginate edible coating improving shelf life of iron fortified food based on pumpkin.

5. Biotechnology as strategy for enhancing nutritional profile of iron fortified foods

Biotechnological processes, which use living organisms and/or derivatives, like enzymes, are being applied for the creation or modification of food products to optimize their functionality.

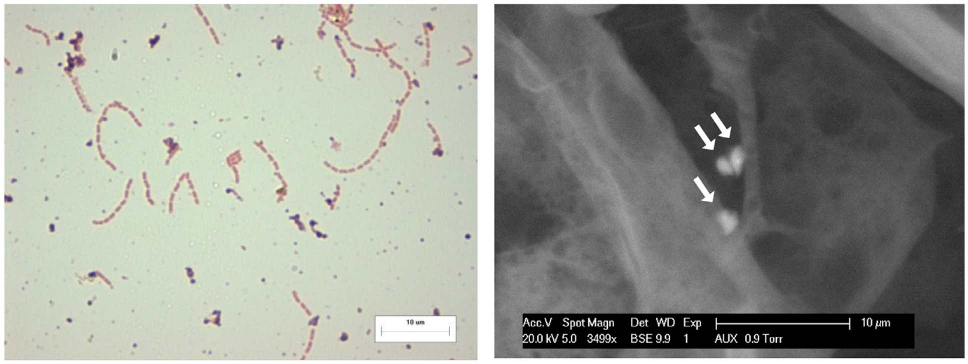

Fig. 4. Micrographs of stained *L. casei* obtained by light microscopy (left, 10 μm bar) and pumpkin tissue impregnated with *L. casei* obtained by ESEM microscopy. Arrows show *L. casei* cells (right, 10 μm bar).

Extensive literature supports the actions of probiotics in maintaining the balance of intestinal flora and it was suggested that a healthy gut could perform a better mineral absorption [60,61]. Dairy products with probiotics have the most developed market up to now. For instance, Silva et al. [62] added iron to a probiotic dairy drink and they studied the effect of mineral fortification on the growth and iron status of pre-schoolers with poor intake of micronutrients. The iron fortified milk beverage was supplemented with *Lactobacillus acidophilus*. They found that children who were fed the probiotic milk beverage exhibited higher red blood cell status and a positive correlation between iron intake and haemoglobin. An increased serum ferritin level was also reported. In addition, nutritional status of the preschool children was improved after intervention [62].

With regards to vegetable matrices, Scheers et al. [63] reported that iron absorption was significantly increased when fermented vegetables with a starter culture of *Lactobacillus plantarum*, replaced fresh vegetables in the low-phytate meal and in the high-phytate meal, while none of the changes in zinc absorption from meals with fresh or fermented vegetables were significant. They observed that lactic-fermented vegetables compared to fresh vegetables increased the ferritin formation and hepcidin release per each mole of iron as the main effect related to the better iron absorption. Moreover, they reported that the phytate level was higher in the fresh vegetables compared to the fermented ones. Genevois et al. [64] formulated a fermented dairy-free dessert using a novel food ingredient based on a pumpkin by-product and containing *Lactobacillus casei* (ATCC®393™) and soy milk. A significant reduction in phytic acid content during fermentation and storage of the final product was reported, which was related to the presence of phytases in *L. casei* [64,65]. Khodaii et al. [66] reported that lactic acid fermentation of bread with *Lactobacillus acidophilus*, either fortified or non-fortified with iron, increases ferritin formation in the intestinal cells, which may be due to the production of acid by lactic acid bacteria as well as phytase activity of the probiotic.

The impacts of phytic acid in foods have become a major concern due to its negative effect on mineral bioavailability and protein digestibility in human nutrition by forming complexes with these compounds. The functional role of phytase is its ability in preventing the antinutritional effects of phytic acid and its salts (phytates), which means that the phytase enzyme increases the bioavailability of the nutrients with great importance in body physiology and general health [67,68]. Both endogenous and exogenous phytases are present in plants, animal tissues and microbial sources (bacteria, yeasts and molds) [69,70]. Nevertheless, phytases from microbial origin may achieve complete phytate degradation, while endogenous phytases just reduce phytates by 73%–80% [71]. Microbial phytases exhibit desirable activity profile over a wide pH range, excellent thermal stability, and broad substrate specificity, they are more promising nutritionally and economically. Thus, the inclusion of exogenous phytase, *i.e.*, microbial phytase in food medium has been seen as a promising solution to improve iron and others bivalent minerals bioavailability [67].

On the other hand, other probiotic strains present low phytase activity, as the case of *Lactobacillus plantarum* Lp299. This probiotic strain was tested in the fermentation of oat gruel with high phytic acid content and it could not be possible to register phytase activity, nevertheless the iron absorption from the Lp299 fermented oat gruel was significantly higher compared to all the other control products [71]. When a fruit drink with low phytic acid: iron molar ratio was fermented with Lp299, similar improvement in iron absorption was reported [72]. In this case, the authors concluded that the positive effect on iron absorption was a result of the live Lp299v, and not the fermentation *per se*, pH or organic acids addition. It was highlighted the importance of intestine colonisation by probiotics. Nevertheless under aerobic growth conditions the intracellular ferrous ion is unstable and in the presence of oxygen some reactive species are created, such as hydrogen peroxide (H_2O_2), superoxide (O_2^-), and hydroxyl radicals ($OH^\bullet$), leading to a deleterious effect on lipid membranes, which is responsible for cell microorganism toxicity [61,73]. Therefore, the viability of probiotics could be affected by iron presence in the same food matrix.

6. Stabilizing probiotics cells in iron fortified food matrices

The production of fermented dairy products, especially yogurt and fresh cheeses, fortified with probiotic bacteria was the first and a stable option for the introduction of probiotics microorganisms into the diet. Fermentation of plant-based material with specific strains of probiotics might render a stable product (as the probiotic culture would be adapted to the food matrix); however, it must be taken care of fermentation because it might change the sensorial properties in a negative way. The use of plant material as carrier (*i.e.*, without fermentation) requires a probiotic strain with great stability during storage [74]. In this sense, *Cucurbita moschata* Duchesne ex Poiret (pumpkin) was enriched with *Lactobacillus casei* (ATTC®393™) [75]. Fig. 4 shows probiotic cells impregnated in pumpkin tissue.

The application of edible coating based on agar – agar was assayed in that opportunity in order to extend the product shelf life. It could be verified that the application of an EC based on agar - agar extended up to 14 days (8 °C) the shelf life of the pumpkin impregnated with *L. casei*. The pH reduction and drying processes contributed to prolong the storage time, reaching 18 days at 20 °C with a viability of the probiotics around 10^8 CFU/g product. In turn, a loss of colour was observed possibly due to the pigments degradation.

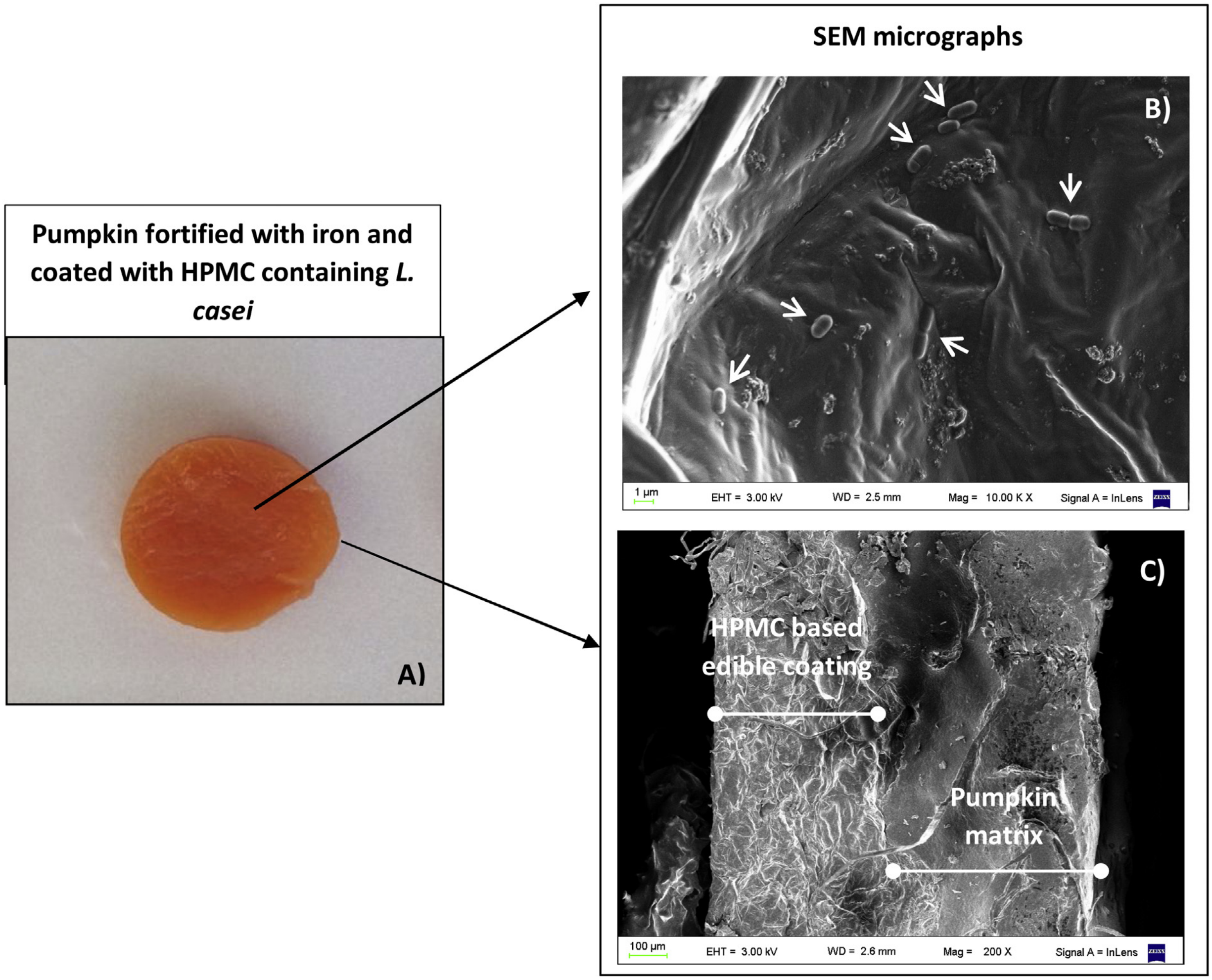

Fig. 5. **A)** Picture of pumpkin snack fortified with iron and coated with HPMC based EC containing *L. casei*. Magnifications of **B)** surface and **C)** cross section of pumpkin obtained by scanning electron microscopy (SEM). Arrows show *L. casei* cells [23].

The use of food hydrocolloids to stabilize bioactive ingredients was widely studied. In particular, pectin was used to produce microencapsulated iron beads [76]. More recently Ghibaudo et al. [77] covered magnetite nanoparticles with pectin composite containing *L. plantarum* achieving a protection for the probiotic against the possible damage by iron reactivity. The freeze-dried ingredient was stable for 60 days at 4 °C.

In the case of combined enrichment of vegetable matrices with probiotics and iron, the dry infusion process can be used to incorporate the mineral into the tissue and, in addition, coating with a food hydrocolloid-based composite containing the probiotic, stabilised the final double fortified product. HPMC coating containing *L. casei* was studied to formulate an iron fortified snack based on pumpkin [75]. Two kind of HPMC based coatings were evaluated, one with 28%-30% of methoxyl groups (E4M) and another one with 19%-24% of methoxyl groups (K4M). Initially, the cell count of *L. casei* and the percentage of bioaccessible iron in the intestinal lumen (55%–70%) did not show significant differences between the studied systems, therefore the type of hydrocolloid and the drying process did not affect these parameters. Nevertheless, at 14 days storage (8 °C), a lower stabilization capacity of *L. casei* was observed for the systems covered with K4M gel, the less branched HPMC.

Fig. 5A shows the ready-to-eat snack based on pumpkin fortified with iron and coated with HPMC based EC containing *L. casei*. In Fig. 5B it is possible to observe the micrograph by SEM of the snack surface. The probiotic cells can be observed into the biopolymeric matrix and the adequate adherence of HPMC based EC to the vegetal matrix is also remarked on Fig. 5C.

Iron fortification is commonly associated with undesirable changes in colour and the metallic taste which makes difficult the consumer's acceptance [78]. Therefore, a triangle test sensory evaluation was performed in that opportunity in order to determine if panellist were able to notice iron presence [79]. The pumpkin with and without iron fortification; both covered with HPMC based EC containing *L. casei* were given to panellist for evaluating and no sensory differences were perceivable due to iron fortification ($P>$ 0.05). Moreover, Affective Test of both samples was performed and no significant differences ($P>$ 0.05) were established for the overall acceptability between system with and without iron, receiving a punctuation above 5 ± 1 on a seven point scale being rated as "like slightly" [80].

7. Conclusion

Since iron deficiency still continues to be a worldwide health problem, food fortification will be an effective long-term approach to improve the iron status of populations. Despite the advances achieved till the present, the evolution in consumer's habits without improving some micronutrients intake, in particular, iron; bring to researchers and manufactures the need to continue innovating to develop novel alternatives in this field. Pumpkin tissue is an adequate raw material for functional food development, and edible coating technology has demonstrated to be a useful strategy to compartmentalize ascorbic acid as well as microorganism probiotics, preserving and/or improving organoleptic and nutritional characteristics of functional foods.

Declaration of Competing Interest

There are no conflicts to declare.

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