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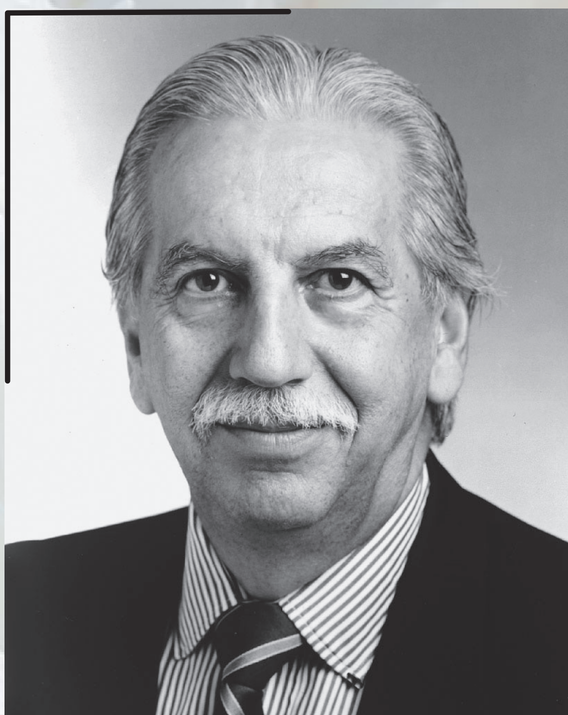

IN MEMORIAM

ARTURO GÓMEZ-POMPA

1934 – 2025

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Cover: Dr. Arturo Gómez-Pompa. *In memoriam*.
1934-2025
Botanist and one of Mexico´s most distinguished scientists

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Agrociencia

FREE-RANGE PIG FARMING AS AN ALTERNATIVE FOR SMALL-SCALE FARMERS IN MEXICO

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ABSTRACT

Backyard pig farming provides income for family production units. The implementation of free-range pig farming systems (FRPFS) improves animal health and well-being, as well as meat quality. In addition, it reduces the environmental impact and dependence on external inputs while increasing profitability through lower production costs and greater access to specialized markets. A review was conducted to identify the FRPFS characteristics that enable and support the implementation of regenerative livestock farming within small-scale production systems. Findings showed that pigs are able to absorb nutrients from tree foliage and grassland plants. Nonetheless, to facilitate digestion, these feeds must be high in protein and low in fiber, requiring the addition of grains or subproducts. In grazing systems, it is necessary to allow long resting periods for the land, since pigs root and disturb the soil. However, their excreta help improve its physical, chemical, and biological properties. FRPFS can be integrated with agricultural systems to strengthen the resilience of small-scale farmers while providing meat with special organoleptic characteristics, which helps produce different products for niche markets at better prices.

Keywords: grazing pigs, regenerative livestock farming, animal welfare, sustainable livestock farming.

INTRODUCTION

In 2017, Mexico produced 1 925 364 Mg of dressed pork. This amount represents the highest value recorded in recent years. However, this figure fell by 22 % during the 2018 change of administration. Following this decline in production, a gradual recovery trend of 1–2 % per year has been observed, culminating in a production level of 1 686

802 Mg in 2021. Out of the entire country's national pork production, 85 % is carried out in semi-intensive and intensive systems, while the remaining 15 % is carried out in backyards (SIAP, 2022). In Mexico, there are 1 046 488 backyard pork production units registered. The state of Veracruz is the most representative of this production system, with 224 598 units. This small-scale production system contributes to the well-being of farmer families by generating income to help cover unforeseen expenses, and it is an activity frequently carried out by women (Solís-Tejeda *et al*., 2024).

Small-scale pork production presents challenges to guarantee sustainability. For instance, backyard production is carried out in pens with concrete floors, which requires constant water cleaning and produces unpleasant odors from manure. As a result, contaminated wastewater is produced and discharged into drains unsuitable for these effluents or onto agricultural land. This causes pollutants, such as nitrites, to infiltrate into water tables, causing negative environmental effects (Solís-Tejeda *et al*., 2022). Although pork has a lower carbon footprint than beef, accounting for only 9 % of greenhouse gas emissions, it is the activity that causes the most acidification in water tables. Additionally, it causes the eutrophication of superficial water bodies due to the discharge of wastewater rich in solid waste, which contains high amounts of nitrogen derivatives (Ottosen *et al*., 2021).

Another challenge for pig farmers is the perception that industrially produced pork is harmful to human health. In addition, there is a growing demand for ethically produced meat, which has led scientists to investigate alternatives such as synthetic meat. Nevertheless, these innovative procedures have not ensured animal well-being, since they still use dubious methods, distancing consumers from a nutritious and natural diet (Moyano-Fernández, 2021). As a response to the challenges of conventional animal production, several paradigms appear, which are contrary to the Green Revolution. Agroecology, holistic livestock farming, and regenerative livestock farming seek to generate products in more natural ways and focus on favoring natural soil processes. These practices help reverse the negative effects of traditional livestock farming, and because they are extensive systems, they produce meat with greater benefits to consumers' health (Velasco *et al*., 2019).

Techniques such as Voisin Rational Grazing (VRG) have been shown to help restore soils by rotating animals in paddocks and practicing ethical management. The use of excrement and urine as organic matter to enrich the soil promotes the richness of edaphic fauna and biological processes that restore the soil (Bautista-García *et al*., 2022). This shows that pigs have productive features that can be implemented in a regenerative livestock farming system, which is feasible for small-scale farmers in Mexico. However, systematizing the available information is necessary for the successful establishment of these systems. Therefore, the aim of this study was to create a review of information about the characteristics of free-range pig farming systems (FRPFS) in order to better understand the advantages and difficulties of implementing this livestock technology in small-scale systems in Mexico.

MATERIALS AND METHODS

The Google Scholar, SciELO, and Scopus databases were consulted to review and synthesize the current knowledge on free-range pig farming The search terms used in English and Spanish were "outdoor pig production" (15 800 results), "pig production in grazing systems" (11 300 results), "free-range pig farming" (16 320), and "extensive pig production" (7 920). As an exclusion criterion, studies published more than 10 years ago were discarded (except for those considered mandatory references and relevant to the development of the sections established in the article), as well as gray literature and works on non-domestic pig species.

The selected articles were clustered by topic, considering relevant topics to propose FRPFS as a current alternative for farmers. These topics included the production system's characteristics, pig productivity parameters in FRPFS, feeding opportunities, feed used, soil and meat quality effects, and system challenges. The "snowball" methodology was used, in which one bibliography leads to others that help to understand the proposed system.

RESULTS AND DISCUSSION

Characteristics of free-range pig farming systems (FRPFS)

The FRPFS is a system in which animals are kept "free" in plots enclosed by fences. This system is based on grazing plot management and the contribution of pastures to animal feeding. With this system, investment capital is reduced by 40 to 70 %, as pigsties and other costly infrastructure are not required. Only shelter huts need to be considered to protect the animals from weather conditions, particularly piglets, so the installation of rustic farrowing huts with heat lamps, insulating materials, and curtains is recommended to protect them during the early stages of life (Parsi *et al*., 2016).

Outdoor production attempts to simulate the natural conditions of pigs and promotes their inherent behaviors. In this system, pigs can spend up to 6 h a day grazing and rooting. They cool themselves by creating nests in the soil, cover their skin with mud for protection against parasites and the sun, and are encouraged to explore using their sense of smell. It also allows pigs to group together and display positive social behaviors. These behaviors are linked to animal welfare, as they reduce fights between individuals, making techniques such as tail docking unnecessary (Pietrosemoli *et al*., 2020).

The FRPFS is a feasible alternative for small-scale farmers since it is more accessible for installation (Parsi *et al*., 2016). In addition, farmers have the opportunity to reduce commercial competition by differentiating their product and accessing new specialized agroecological markets. This benefits from a new trend of consumers that perceive the field system as a favorable way of production for the environment and the well-being of the animals (García-Madrid *et al*., 2021).

It is important to consider that this system requires animals with greater hardiness than industrialized farm pigs, since they are directly exposed to environmental conditions. Creole and mixed-race pigs have better resistance to diseases and external environmental factors, as well as a greater ability to digest low-quality feed. Backyard pig farming is an opportunity to breed animals with greater adaptive genetic strengths, which may be beneficial for their adaptation in the field (Martínez-Velázquez *et al*., 2016).

The Mexican Hairless Pig (MHP) is a hardy species that lives in warm tropical regions. It is a creole variant that originated from Iberian pigs (*Sus scrofa mediterraneus*) and has adapted to the extreme conditions of the tropics, such as high relative humidity, parasites, and high temperatures. However, it is hardly appreciated by butchers, since it accumulates excess fat after 70 kg. Despite this, it is an opportunity to generate novel products in which their specific characteristics that differentiate them from intensive-production pigs, such as their intense flavor and soft meat, can be exploited (Ramos-Canché *et al*., 2020).

The MHP may accumulate large amounts of fat within and on its carcass, which is very important in the production of high-quality, high-value meat products such as cured hams (Delgado *et al*., 2002). This fat is of higher quality and may have beneficial properties for human health since it comes from animals that eat a variety of plants, and it can be controlled by restricting concentrated feeds and the age of slaughter.

Feeding opportunities in free-range pig farming systems

The use of balanced feeds for animals has been limited due to various circumstances in the global market that raise prices. Small-scale pig farming is an economic challenge, since the production costs tend to be higher than those for semi-intensive and intensive systems. In addition, the sale price established by local markets does not usually cover these costs (Solís-Tejeda *et al*., 2024). Balanced feeds represent between 60 and 70 % of the production costs for small-scale farming, so the use of green feeds available in farmers' plots is an alternative to reduce the consumption of concentrates.

Green feeds of excellent quality, which provide proteins, minerals, vitamins, and energy, are available (Velasco *et al*., 2019). However, pigs are inefficient in utilizing fibrous feeds since they are monogastric; they cannot perform pre-gastric fermentations like ruminants, nor do they have enzymes that help degrade fiber. Despite this, when pigs eat forage, they can assimilate proteins through fermentation in the cecum and the colon of the large intestine.

When pigs are fed high-fiber diets, they modify their microbiota and produce more cellulolytic bacteria in the gastrointestinal tract (González *et al*., 2020). This way, they acquire the ability to use half the hemicellulose consumed and a little less than one-third of the cellulose. Only lignin cannot be digested; therefore, tender pastures, which contain a lower amount of this compound, are better for feeding pigs (Savón, 2002). After the pasture is consumed, fermentation in the large intestine produces volatile fatty acids (VFA), such as acetic, butyric, and propionic acids. The mucosa of the cecum and colon can efficiently transport these nutrients. The VFAs derived from the fermentation of pastures can cover between 5 and 20 % of the animals' energy requirements (Milera-Rodríguez, 2022).

Another characteristic worth considering is the volume of the pigs' gastrointestinal system. In comparison with ruminants, the amount of dry matter from pastures they can process is limited, since they are full before satisfying their nutritional requirements. This is worse in piglets and developing pigs with digestive systems with lower capacities and fewer microorganisms able to digest fiber. Therefore, grazing is more common in gestating and lactating females (Parsi *et al*., 2016).

High-fiber diets in pigs have been shown to reduce nutrient assimilation, decrease flesh weight gain, and increase gut weight. This depends on the soluble-to-insoluble fiber ratio provided, so the pigs should not be fed only pastures. Diets must include easily digestible concentrated elements rich in nutrients that complement the pastures (González *et al*., 2020). On the other hand, a diet with fiber promotes the health and well-being of pigs during gestation and labor, since fiber reduces nervous movements in the sows related to symptoms of discomfort in the days before and after labor (Ramos-Canché *et al*., 2020).

Nutritional characteristics of feeds used

Forage, particularly legumes, is a source of protein that pigs can absorb, and their low lignin content facilitates its assimilation (Table 1). Additionally, it provides minerals such as iron (Fe), which is crucial for the development of piglets in their first stages of development for the formation of hemoglobin. As in conventional systems, it is

Table 1. Nutritional composition of pastures consumed by pigs in free-range production systems.

Pasture	DM	CP (%)	NE$	NDF (%)	LIG (%)	Reference
Alfalfa (*Medicago sativa* L.)	89.7	16.7	NI	45.0	6.5	Araiza-Ponce *et al*. (2020)
Rye grass (*Lolium perenne* L.)	16.2	22.1	1.4	44.6	NI	Cardona-Iglesias *et al*. (2020)
Red clover (*Trifolium pratense* L.)	NI	21.5	NI	27.0	NI	Vallejos-Fernández *et al*. (2021)
White clover (*Trifolium repens* L.)	NI	23.5	NI	23.4	NI	Vallejos-Fernández *et al*. (2021)
Forage wheat (*Triticum aestivum* L.)	NI	17.2	NI	54.7–64.9	NI	Zamora-Villa *et al*. (2016)
Forage oat (*Avena sativa* L.)	27.5	8.6	1.3	47.0	NI	Mamani-Paredes and Cotacallapa-Gutiérrez (2018)
Forage peanut (*Arachis pintoi* Krapov)	NI	24.0	NI	52.5	2.9	Rodríguez *et al*. (2010)

MD: Dry matter; CP: crude protein; NE: net energy; NDF: neutral detergent fiber; LIG: lignin; NI: not indicated.
$Mcal kg^{-1} of DM.

recommended to add iron intravenously, since an iron-rich diet or supply during gestation does not fulfill the iron needs of piglets (Velasco *et al*., 2019).

On average, the voluntary intake of grasses by pigs weighing between 10 and 20 kg fluctuates between 0.08 and 0.1 kg of dry matter (DM) d^{-1}, accounting for 9.5 % of their diet. This value increases slightly for animals weighing 40 to 60 kg at an estimated intake of 0.208 to 0.237 kg DM d^{-1}, accounting for 10.2 % of their diet. This occurs when a balanced feed is supplied with a low restriction of 15 % (Soledad-Carballo *et al*., 2010).

By restricting the availability of concentrate or balanced feed, pigs increase their intake of pasture, and production costs decrease. However, it is important to establish a diet that incorporates grain byproducts like wheat semolina, animal protein like whey, and energy sources such as potato, taro, yuca, and banana. Farmers can generate these complementary feeds in the production unit. Depending on the ingredients used, the fattening time may be longer than expected in the conventional diet, but the farmer's dependence on the purchase of inputs is reduced, improving its profitability (Soledad-Carballo *et al*., 2010; Milera-Rodríguez, 2022). Thus, the intake of pastures depends on the age of the animals and the quality of the grass (González *et al*., 2020); however, it must be complemented (Table 2).

Although FRPFS are an alternative to the impact of pen production, it is necessary to analyze the factors that contribute to the degradation of grass and forage-based farming

Table 2. Grazing techniques and complementary feed in pigs by race, weight, and type of feed (F).

Race	Weight (kg)		F	Cycle (d)	Complementary feed	Grazing technique	Reference
	In	Fin					
Pampa Rocha	10	20	f1, f2, f3	25	CM (75 %), MBM (6.5 %), SBM (17.5 %), NaCl (0.5 %), vitamins and minerals (0.5 %)	Rotational grazing in 300 m^2 strips, 187.5 m^2 per animal	Soledad-Carballo *et al*. (2010)
Duroc × Large White	40	57	f1, f2, f3	25	CM (75 %), MBM (6.5 %), SBM (17.5 %), NaCl (0.5 %), vitamins and minerals (0.5 %)	Rotational grazing in 300 m^2 strips, 187.5 m^2 per animal	Soledad-Carballo *et al*. (2010)
Mestizos (Landrace)	W	70–80	f4, f5, f6, f7	180–210	Corn grain, soybean hulls, rice meal, vitamin supplement, salt, and calcium	Continuous grazing, last 30 d in confinement with commercial feed	Velasco *et al*. (2019)
Gascón	W	170	f2, f4, f8	360–720	Acorns, chestnuts, wheat, oat, barley, rye, triticale, fava bean, peas, colza, or sunflower bran	Grazing system with forest, 500 m^2 per animal	Lebret *et al*. (2021)

In: initial; Fin: final; W: weaning; f1: chicory (*Cichorium intybus* L.); f2: red clover (*Trifolium pratense* L.); f3: annual ryegrass (*Lolium multiflorum* L.); f4: white clover (*Trifolium repens* L.); f5: perennial ryegrass (*Lolium perenne* L.); f6: birdsfoot trefoil (*Lotus corniculatus* L.); f7: orchardgrass (*Dactylis Glomerata* L.); f8: Bermuda grass (*Cynodon* sp.); CM: cornmeal; SBM: soybean meal; MBM: meat and bone meal.

systems. This includes inadequate management of grazing (overgrazing caused by a mismanaged rotation), low-quality pastures, few trees in pens, and insufficient water provided for the animals (Bautista-García *et al*., 2022). A more agroecological approach is necessary, increasing the diversity of plants that are resistant to pests, weed management, and tree-grass integration.

Tree shades provide habitats for various insect species by creating microclimates that encourage their development. They also promote complex relations between plant-eaters and bioregulators, benefitting pollinators, coprophages, and decomposers of organic matter, which help maintain the biological stability of the plots (Milera-Rodríguez, 2013). It is feasible to integrate trees that enhance the stability of the system by capturing nitrogen products in lower soil levels resulting from livestock activity, and using their leaves and fruit as animal feed, which farmers can harvest and provide to the animals, which is particularly important during the dry season (Sarmiento-Franco *et al*., 2022).

Trees also provide other advantages, such as animal protection with shade, soil conservation, water storage, and the extraction of minerals from the deepest layers in the soil to the top layer by means of the leaves (Araiza-Ponce *et al.*, 2020). Therefore, it is convenient to integrate trees of high nutritional value into the FRPFS to optimize the diets of the animals (Table 3), the chemical composition of which has been used for its nutritional capacity in the feeding of pigs.

Table 3. Nutritional composition of foliage and tree seeds used to feed pigs in free-range production systems.

Tree	PP*	DM (%)	CP (%)	NDF (%)	LIG (%)	Reference
Ramón (*Brosimum alicastrum* Swarth)	F	40–44	13–17	36–46	3.8–7.9	Sarmiento-Franco *et al*. (2022)
Ramón (*B. alicastrum* Swarth)	S	92	12.8	NI	NI	Sarmiento-Franco *et al*. (2022)
Leucaena (*Leucaena leucocephala* Lam.)	F	89.5	21.3	42.9	8.1	Araiza-Ponce *et al*. (2020)
Moringa (*Moringa oleífera* Lam.)	F	17–25	14–22	35–54	NI	Rivero *et al*. (2020)
Morera (*Morus alba* L.)	F	30.8	14.6	37.8	NI	Rodríguez-Molano *et al*. (2019)

DM: dry matter; CP: crude protein; NDF: neutral detergent fiber; LIG: lignin; NI: not indicated. *Estimated content in foliage (f) or seeds (S).

The effect of replacing concentrated feed with tree leaves is similar to that of grasses, with reduced nutrient assimilation as the percentage of leaf consumption increases. In this regard, *Brosimum alicastrum* Swarth leaves are estimated to provide 1.3 Mcal kg^{-1} of DM of metabolizable energy to pigs, whereas seeds provide 3.7 Mcal kg^{-1} of DM of raw energy (Sarmiento-Franco *et al*., 2022). Pigs can digest fresh *Leucaena leucocephala* Lam. leaves by 60 % of DM, 57.8 % of the organic matter, and 66.5 % of raw NDT; they can also assimilate calcium and phosphorous (Araiza-Ponce *et al*., 2020).

Fresh mulberry (*Morus alba* L.) leaves are a good replacement for Soybean meal and Corn, since they contain a percentage of protein that can surpass 20 % and digestibility values in pigs between 75 and 85 % (Rodríguez-Molano *et al*., 2019). In a study that replaced the soybean meal in the balanced feed (usually standardized with 20 % soybean meal) and supplemented 40 % of the dry base of the diet with mulberry foliage, a daily weight gain of 0.473 kg was recorded, 0.129 kg less than the control diet. Intake was lower with the moringa diet (1.5 kg MS d^{-1}) in comparison with the control (2 kg MS d^{-1}); however, feed conversion was better (3.1) in contrast to the control diet (3.4) (Rivero *et al*., 2020). Mulberry improves digestibility in pigs when stems are removed and only the leaves with petioles are provided (Rodríguez-Molano *et al*., 2019).

Along with the benefits mentioned earlier, producing some of the ingredients for animal feed reduces dependence on inputs and the carbon footprint of livestock farming. The further the distance covered to supply the ingredients to produce the balanced pig feed, the greater the carbon footprint (Ottosen *et al*., 2021).

Soil conditions in free-range pig farming systems

The flow of rainwater from mountains to oceans has increased in speed because soil and plant-covered surfaces that can retain water have reduced. This decrease is due to the soil degradation caused by urbanization and farming malpractices, giving way to drought and flood periods (Suárez-Castillo, 2018). On the other hand, the oxidation of organic matter generated in conventional farming releases CO_2 and CH_4 from the soil to the atmosphere, warming the planet and reducing the soil's productive capacity (Ottosen *et al*., 2021).

Animal production through regenerative livestock farming can improve soil conditions when integrated with soil fauna, increasing porosity, organic matter, carbon sequestration, and water retention at low costs (Suárez-Castillo, 2018). Pigs are inefficient in the assimilation of nutrients, so they release between 40 and 50 % of nutrients in their excretions, which contain nitrogen, phosphorous, potassium, calcium, magnesium, organic matter, and micronutrients not available in commercial soil-improving products. These nutrients can be deposited in the soil when grazing and incorporated by microorganisms as they are decomposed into humic substances, contributing to soil fertility (Rivero *et al*., 2013).

Grazing techniques in cattle, such as Voisin Rational Grazing (VRG), have been demonstrated to regenerate soils damaged by intensive production, improving soil bulk density. With these techniques, nutrients are reincorporated, and the amount and variety of soil fauna species increase (Bautista-García *et al*., 2022). Additionally, adequate pasture management through VRG helps produce higher-quality forage for the animals and increase the available biomass in comparison with conventional grazing (Suárez-Castillo, 2018). These benefits are obtained without chemical fertilizers, since the organic matter produced by the animals is evenly distributed in the pastures (Trinidad-Santos and Velasco-Velasco, 2016). A high stocking rate over short periods on healthy and biodiverse soils improves nutrient availability and creates a virtuous cycle that benefits both the soil and production (Figure 1).

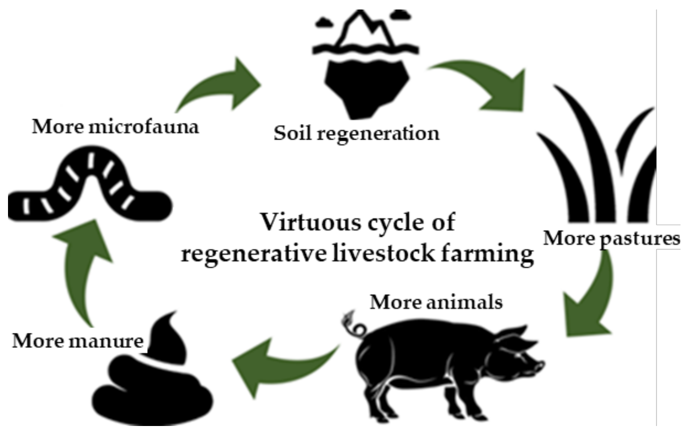

Figure 1: Virtuous cycle of regenerative livestock farming in soil improvement and production efficiency.

To adapt these grazing techniques to pig farming, it is recommended to consider factors such as the division of plots to give pastures a longer resting time and avoid soil erosion while maintaining plant coverage (Milera-Rodríguez, 2022). When three methods for housing pigs on pasture were compared, it was discovered that rotating the animals increased weight gain by 8.5 % and feed efficiency by 8 %. In the field, nutrients such as nitrate, potassium, phosphorous, manganese, zinc, and copper were better distributed when plots were rotated or animals were moved in strips, which favored plant recovery by 22 %, while the externalities of conventional systems were not generated (Pietrosemoli *et al*., 2020).

It is also important to consider the natural behavior of pigs. Rooting, which is the habit of pigs to stir the soil with their snouts, often affects pastures and exposes the soil (Milera-Rodríguez, 2022). A poorly planned outdoor pig farming system can degrade the vegetative cover, exposing the soil to erosion, runoff, and compaction (Bautista-García *et al*., 2022). In a 174 d experiment, at a density of 6 kg m^{-2} of pigs in a continuous grazing system, the amount of total available organic carbon in the soil ranged from 3.2 to 4.3 mg g^{-1} in the first 5 cm of depth; total phosphorous increased from 100.1 to 168.6 mg kg^{-1} at the same depth; the carbon content in humic acids increased from 0.7 to 0.8 mg g^{-1} from 5 to 10 cm depth; and the carbon content in fulvic acid increased from 0.029 mg g^{-1} to 0.34 mg g^{-1} (Rivero *et al*., 2013).

Pig manure is commonly used in agriculture for having a more nitrogen-rich chemical composition (3–5 %) than cattle manure (1–3 %). In general, Mexican soils are degraded and low in organic matter, with only about 1 %. This type of soil is estimated to provide 17.4 kg of N ha^{-1}, in contrast to a fertile soil (4 % of organic matter), which can provide 69.6 kg of N ha^{-1}. Therefore, pig excretions have a great potential to regenerate organic matter in the soil (Rivero *et al*., 2013; Trinidad-Santos and Velasco-Velasco, 2016).

The possibility of using fertilized grazing pens is suggested for agricultural production during the resting period of the plot, since grain yield, number of ears, specific grain weight, and final plant height have been proven to be similar between chemical and organic manure-based fertilizers on maize crops, leading to savings on agricultural inputs for farmers and favoring the resilience of the small-scale agricultural and livestock activities (Couoh-Moo *et al*., 2022). Along with this is the benefit of the work of the animals in plowing as a result of rooting, which mixes the nutrients, loosens the soil, and prepares it for sowing (Milera-Rodríguez, 2022).

Meat quality in free-range pig farming systems

The use of chemical additives in pig feed, such as ractopamine hydrochloride, antibiotics, hormonal implants, and β-agonists, has raised concerns among the health sector and consumers in Mexico (Almaguel *et al*., 2021). On the other hand, FRPFS is a more natural way of producing meat without additives, which leads to a greater innocuity of the final product (Velasco *et al*., 2019).

Pork is nutritious and high in cholesterol, zinc, iron, and vitamin B12. Pork may improve its properties depending on the diet of the animal. A study conducted with people that ate 100 g of pork fed with 10 % avocado meal for six weeks showed a reduction in body fat (1.3 %), body mass index (BMI) (1.3 %), visceral fat (0.4 %), waist circumference (4.4 %), and blood glucose (18.5 %) (González-Jiménez *et al*., 2021). Pork from pasture-fed animals contains high levels of unsaturated fatty acids, such as oleic acid (Velasco *et al*., 2019). However, these nutritional advantages will depend on environmental conditions and the quality of the animals' diet.

Pigs that finish fattening in winter exhibit a higher fat thickness and a more basic hydrogen potential (pH) in the meat, which allows for a longer shelf life after processing (Lebret *et al*., 2021). Meat under this production system dehydrates more slowly, which favors its industrialization and makes it possible to produce different cured products such as cold meats. Product transformation is key to competing with conventional production, which is preferred by butchers due to its higher carcass yield and low-fat content (Ramos-Canché *et al*., 2020). Small-scale farmer associations can help broaden the productive area in which an FRPFS can be implemented and collectively increase livestock benefits.

Challenges of the system

The FRPFS presents challenges that may reduce farmers' interest in its use. To establish these systems, a greater extension of land is required, unlike the conventional system, since the animal load is low (Pietrosemoli *et al*., 2020). In Mexico, small-scale pig farmers usually have plots smaller than 1 ha (Solís-Tejeda *et al*., 2024). Likewise, weight gain in these systems tends to be lower, so it is necessary to provide varied diets with adequate energy and protein levels to reduce the fattening period (Soledad-Carballo *et al*., 2010; Lebret *et al*., 2021).

Despite this, disease reduction must be taken into consideration, since management with less stress improves profitability (Milera-Rodríguez, 2022), and the integration with other subsystems such as maize favors the acceptance by agricultural farmers, who require reductions in fertilization costs (Couoh-Moo *et al*., 2022). On the other hand, animals that have been adapted to conventional systems can undergo stress and diseases when placed in the outdoors. It is important to integrate rustic animals, such as Creole pigs, or to consider a generational adaptation of the herd to the system (Ramos-Canché *et al*., 2020).

CONCLUSIONS

The free-range pork production systems are viable for small-scale farmers, since they reduce dependence on external inputs, increase the resilience of the production units, and favor animal well-being by allowing natural behaviors. In these systems, pigs produce fewer negative externalities, and grazing can be managed to ensure their excretions benefit the regeneration of organic matter in the soil. Commercialization requires different channels than those for conventional pork, but the benefits must be comprehensively quantified, and the interaction with other agricultural and forestry systems should be leveraged. Research on how to increase daily weight gain, adjust stocking rates, and optimize rotation to improve soil management is recommended.

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BARBACOA PRODUCERS' PERCEPTIONS OF ANIMAL WELL-BEING AND ADEQUATE SLAUGHTERING PRACTICES IN SLAUGHTERHOUSES AND THE MUNICIPAL ABATTOIR IN CAPULHUAC DE MIRAFUENTES, MEXICO

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ABSTRACT

This work examines the importance of the perception of good practices in the slaughtering and management of sheep in Capulhuac, State of Mexico, in terms of animal well-being, meat quality, and food security. Meat contamination can originate in slaughterhouses due to inadequate facilities, poor personnel practices, and inefficient maturation times. These factors produce stress in the animals, affecting the sensory characteristics of the meat (texture, color, and water retention) due to biochemical alterations, such as abnormal *post-mortem* pH levels. Therefore, the main actors of the sheep meat production chain (slaughterers and barbacoa producers) were interviewed. The information was evaluated using a discriminant analysis, a principal components factorial analysis, and a cluster analysis to characterize and cluster the perceptions. The results indicate that interviewees consider management before and after slaughtering and animal well-being as critical factors, relating these aspects to the quality of the meat. In addition, they consider that the education of operators in animal welfare is fundamental, along with the promotion of practices that ensure suitable living conditions for animals, such as adequate diets, freedom to move, and a lack of stress. Animal welfare affects the final product's quality and reflects an ethical commitment in production. Likewise, the need to improve slaughterhouse working conditions, such as implementing regulations that ensure humane treatment for animals during their raising and slaughter, is highlighted. This is critical to maintaining the competitiveness of the meat produced and meeting consumers' growing expectations for sustainable and ethically obtained products.

Keywords: Adequate slaughtering practices, animal well-being, food security, slaughterer, meat quality.

INTRODUCTION

Animal protein consumption has played an important role in the evolution of the human diet around the world. Meat contains the nine essential amino acids, including tryptophan, threonine, and lysine, which participate in the formation of bodily structures and the performance of vital biological functions (Njoga *et al*., 2023). FAO (2023) classifies it as a source of high-quality protein, recommended for inclusion in the human diet to maintain a healthy lifestyle when consumed in moderation, in a proportion of 0.75 g per kilogram of body weight per day. In addition, meat provides energy benefits due to its fatty acids, and its content of vitamins and minerals promotes several biological functions of the organism. However, it is crucial to verify that all processes before obtaining the meat are adequately carried out, since its composition can easily be contaminated with microorganisms and become a vector of food-transmitted diseases (Schirone *et al*., 2019). This highlights the importance of identifying and mitigating sanitary risk factors throughout the production chain (Mendonça *et al*., 2019).

Meat production, particularly lamb barbacoa, is the main economic activity in the municipality of Capulhuac de Mirafuentes, State of Mexico, Mexico. Currently, nearly 70 % of its active population participates directly or indirectly in this production chain, including the introduction of animals, slaughtering of sheep, and commercialization of carcasses, as well as the production and distribution of barbacoa, generating local resources and promoting the interaction with external production chains within the country (Pulido *et al*., 2018; Herrera-Haro *et al*., 2019).

Despite being a small municipality, Capulhuac de Mirafuentes is known for providing the metropolitan area of the Valley of Mexico with three main products on weekends: barbacoa, lamb tripe, and broth. Barbacoa is obtained by steaming lamb meat in an underground hole, covered with bricks and pulque agave (Espejel-García *et al*., 2015; Pillado-Albarrán *et al*., 2017; Vázquez-Martínez *et al*., 2018). Lamb *pancita* is prepared using the animal's viscera, finely chopped and mixed with epazote, onion, garlic, dried chilies, and spices. This mixture is stuffed into the lamb's stomach and steamed in a stone oven or stockpots, along with barbacoa. Finally, the broth is prepared from a mixture of water, chickpeas, dried chilies, rice, herbs, and seasonings, complemented with juices and fats released during the cooking of the barbacoa and *pancita*.

In this municipality, approximately 400 000 animals are slaughtered every year. However, as in other regions in Mexico, 90 % of the slaughtering destined for human food is carried out in municipal and private slaughterhouses, which often do not meet the necessary conditions to guarantee the well-being of the animal, nor do they have adequate desensitizing processes, which jeopardizes the quality and innocuousness of the meat, as well as being a risk to the health of consumers (Archundia-Velarde *et al*., 2024). Nowadays, aspects related to breeding, transportation, and slaughtering methods, particularly those to ensure the absence of pain in the animals, are evaluated (Gallo and Tadich, 2008; Miranda-de la Lama, 2013; Solórzano *et al*., 2019). The aim of this study was to identify barbacoa producers' perceptions of slaughtering practices and perspectives on animal welfare among workers and municipal meat processors in Capulhuac de Mirafuentes.

MATERIALS AND METHODS

Population studied

The study was aimed at slaughterers that produced barbacoa at the Barbacoa Producers' Association of Capulhuac de Mirafuentes, Mexico. A total sample of 58 individuals was considered based on a simple randomized sampling, considering a finite population, with a trust value of 95 % (Z = 1.96), an accuracy of 5 %, a variance estimator equal to 0.25 [$\sigma 2 = \pi(1-\pi)$] and a value of N = 65, which accounted for 85 % of the association. To minimize error, participation was announced as voluntary, and producers were assured that establishment owners and slaughterers were unaware of the sampling day, as well as that there would be no economic retribution for participating establishments, indicating that all information would be kept confidential and used solely for research. The study was conducted from February to May 2022.

Survey design

All information was gathered by conducting face-to-face surveys with slaughterers and barbacoa producers who work at the municipal meat processors and sheep producers. The structure of the questionnaire was validated by experts on the topic in the Faculty of Veterinary Medicine and Zootechnics of the Autonomous University of the State of Mexico. The survey included 22 descriptors related to the slaughtering and the production of barbacoa. The questions were focused on the producer's perception and were measured using an ordinary scale consisting of five points: 1, definitely not; 2, probably not; 3, indifferent; 4, probably yes; and 5, definitely yes.

Statistical analysis

Two multivariate statistical techniques were used. Based on the survey data collected, a principal components analysis was conducted for factor extraction, along with the Kaiser-Mayer-Olkin (KMO) index and Bartlett's test of sphericity to measure the correlation among variables. Variables with communality ($h < 0.9$) were not included in the factorial analysis factor, since these variables were not correlated with the new factors. The selected factors had eigenvalues ≥ 1.

To better understand the factors identified, an orthogonal rotation method (Varimax) was carried out. The scores were estimated using the regression method and kept as new variables. A hierarchical cluster analysis was performed to identify similarities and differentiate the perceptions of the slaughterers. The squared Euclidean distance was used as a similarity measure, and clustering was performed using Ward's method. To select the most significant variables and differentiate the clusters obtained, the Kruskal-Wallis non-parametric test and the Mann-Whitney test were performed. Analyses were performed using SAS v15.0 (SAS Institute Inc., 2002).

RESULTS AND DISCUSSION

The physical and mental conditions of the animals during their raising and transportation, along with the stages before and after slaughtering, which include the resting period, the desensitization method, hygiene-sanitary management of the carcass, and maturation, are determining factors to preserve the innocuousness and quality of the meat (García-Díez *et al*., 2023). This approach has gained particular importance among the different actors of the production chain, from health authorities to final consumers, who demand responsible practices that ensure safe, sustainable, and ethically produced foods (Mendonça *et al*., 2019).

Slaughterhouses are a critical link in the production chain of barbacoa, as it takes the transformation of muscle tissue into meat, which is sterile in normal animals under normal physiological conditions at the time of their death (Terlouw and Gagaoua, 2022). Diverse extrinsic factors determine the presence of microorganisms, as well as meat color, flavor, and texture. Among the main risk factors are deficiencies in infrastructure, inadequate equipment operation (Joshi *et al*., 2003; Aguayo-Ulloa *et al*., 2021), poor hygienic practices of the personnel (Ashuro *et al*., 2023), and inappropriate maturing times (Álvarez *et al*., 2022). These problems are particularly relevant in municipal slaughterhouses, where sanitary control and regulatory compliance are frequently limited.

General characteristics of barbacoa producers and slaughterers

Results showed that 100 % of the participants were men, of whom 51.7 % were between 45 to 60 years old, reflecting an economically active middle-aged population (Table 1). Regarding their educational level, 46.6 % reported having finished high school, whereas 27.6 % only finished elementary school, displaying a predominance of basic academic formation. Most interviewees had more than 10 years' experience in slaughtering and barbacoa preparation, which suggests a transmission of practical and traditional knowledge of the craft.

Principal components analysis

Eight principal components (PC) were identified, which explained 73.9 % of the variability of the data (Figure 1, Table 2), and were named depending on the correlated variables.

PC 1. Animal management before and during slaughtering

This factor had the greatest contribution (Table 2), explaining the variance among survey respondents (17.82 %) and involving aspects of the application of regulations on the management of animal well-being before and during animal slaughtering. It was composed of three correlated descriptors: Do you consider that slaughterhouses comply with adequate regulations to slaughter animals? Do you believe slaughterhouses manage animals adequately before slaughtering? Do you believe slaughterhouses manage animals correctly during slaughtering? For barbacoa producers, this phase was a more relevant procedure for workers during slaughtering.

Table 1. General characteristics of barbacoa producers and slaughterers interviewed in the municipality of Capulhuac de Mirafuentes, State of Mexico.

Barbacoa producers	Number of interviewees (n)	Percentage of interviewees (%)
Gender		
Female	0	0
Male	58	100
Age (years)		
18–30	6	10.3
30–45	14	24.1
45–60	30	51.7
>60	8	13.8
Level of studies		
Elementary	16	27.6
Middle	27	46.6
High School	12	20.7
University	3	5.2
Origin		
Urban	2	3.4
Rural	56	96.6
Experience (years)		
1–3	1	1.7
4–6	2	3.4
7–10	12	20.7
>10	43	74.1

Number	Eigenvalue	Percent	Cum Percent
1	3.9196	17.817	17.817
2	2.8509	12.959	30.775
3	2.2103	10.047	40.822
4	2.0062	9.119	49.941
5	1.7437	7.926	57.867
6	1.3291	6.041	63.908
7	1.1310	5.141	69.049
8	1.0709	4.868	73.917
9	0.9339	4.245	78.162
10	0.8224	3.738	81.900
11	0.7533	3.424	85.323
12	0.6644	3.020	88.343
13	0.5047	2.294	90.637
14	0.4182	1.901	92.538
15	0.3423	1.556	94.094
16	0.3229	1.468	95.562
17	0.2829	1.286	96.848
18	0.2225	1.011	97.859
19	0.1829	0.831	98.690
20	0.1480	0.673	99.363
21	0.0951	0.432	99.795
22	0.0451	0.205	100.000

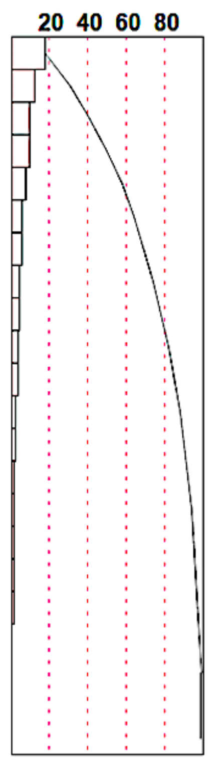

Figure 1. Communality and eigenvalues of the principal components (PC) and their contribution towards the explanation of the variance (%) of the data in barbacoa producers in Capulhuac de Mirafuentes, State of Mexico.

Table 2. Principal components (PC) in the perception of barbacoa producers on the slaughtering of sheep in the municipality of Capulhuac de Mirafuentes, State of Mexico.

PC	Name assigned to the component	Correlated variables	Eigenvalue	Total variance explained (%)	Explained variance accumulation (%)
PC1	Animal management before and after slaughtering	7, 8, 9	3.91	17.81	17.81
PC2	Stress effects on the sensory characteristics of the meat	4, 5, 17, 18, 22	2.85	12.96	30.77
PC3	Knowledge and perception of slaughterers on animal well-being	3, 10, 12, 14	2.21	10.05	40.82
PC4	Regulations of animal well-being and its importance for meat quality	11, 13	2.00	9.11	49.90
PC5	Perception of the existence of emotions in draft animals	1, 19	1.74	7.90	57.86
PC6	Importance of the relation between adequate livestock practices and animal well-being	6, 15	1.33	6.04	63.90
PC7	Importance of animals expressing the five freedoms of animal well-being during transportation to the slaughtering area	16	1.13	5.14	69.09
PC8	Importance of the actors involved in the barbacoa production chain, knowing the regulations of animal well-being	20, 21	1.07	4.86	73.91

PC 2. Effects of stress on the sensory characteristics of the meat

This component had the second-highest contribution to the explanation of variance among respondents (12.96 %). It includes aspects related to the effects of the slaughtering methods on meat quality characteristics, particularly the impact of stress on the animals. It is composed of five variables: Do you believe the methods and/or ways of slaughtering affect the features of the lamb meat? Do you believe that the animals that suffer generate undesirable meat? Do you believe that farm animals should be free of fear and stress? Do you consider that farm animals feel pain? Do you believe that stress in animals during production and transportation affects the quality of meat and its by-products? For this group of barbacoa producers, it is important that the workers use slaughtering methods that conform to regulations and understand that proper management has a positive impact on the meat's properties.

PC 3. Knowledge and perception of slaughterers on animal well-being

This component explained 10.05 % of variance among survey respondents and is composed of four variables related to education and knowledge on animal well-being. These variables range from living conditions in the flock to practices before slaughtering: Do you consider slaughterhouses to be an important link in the sheep production chain? Do you believe animals experience pain, anguish, and fear before slaughtering? Do you think education on animal well-being is necessary for slaughterhouse operators and workers? What is your level of knowledge on the living conditions of farm animals? For this group of barbacoa producers, education on animal well-being is crucial, from growth to slaughter.

PC 4. Regulations on animal well-being and their importance for meat quality
This component explained 9.12 % of variance and covers knowledge on regulation to guarantee animal well-being, from standards of living in the flock to slaughtering practices. It includes three variables: Do you consider lamb to be important for the municipality of Capulhuac de Mirafuentes? Do you believe new laws on animal well-being are necessary to avoid abuse in slaughterhouses? Do you believe stress in animals during production and transportation affects the quality of the meat you eat? This group considers it a priority to review and modify regulations on animal well-being for drivers and producers, ensuring their compliance as per the current demands of the consumer society.

PC 5. Existence of emotions in draft animals
This factor was composed of two variables, which represent 7.9 % of the variance explained. This includes the importance of the sheep slaughterhouses in the municipality (perception of work-income sources), as well as the perception of survey respondents on the emotions felt by farm animals.

PC 6. Importance of adequate livestock practices for animal well-being
This component represented 6.04 % of variance and is based on two variables: the perception of respondents on the animals raised in complete freedom and the perception of the implementation of adequate practices in animal management, feeding, care, and health. In this component, little interest is shown by respondents regarding the different forms of sheep production raised for slaughtering.

PC 7. Importance of animals expressing the five freedoms of animal well-being during transportation to the slaughtering area
This component consisted of only one variable, which represented 5.14 % of variance. As in PC 6, respondents displayed little interest in whether drivers allow animals to express natural behaviors of their species during their transportation from the production area to the slaughterhouse.

PC 8. Importance of knowing the regulations of animal well-being in the barbacoa production chain
This component consisted of two variables that make up 4.86 % of the variance explained. These variables reflect respondents' perception as to whether the available

information in Mexico regarding the well-being of farm animals and the need to implement improvements in their protection in the country is adequate. PCs 6, 7, and 8 had the lowest perception by respondents, with an explanation of 16.05 % of the accumulated variance, while PCs 1 to 5 explained 57.87 %.

For slaughterhouse operators and barbacoa producers, the most important factors influencing the final product are proper animal management before and after slaughter, with a focus on reducing stress during desensitization. Likewise, they also considered the importance of implementing animal well-being principles from an early age as a key factor, promoting conditions that ensure the physical and emotional comfort of the animals throughout their productive life. This integral vision is consistent with recommendations by international organizations such as the World Organization for Animal Health (WOAH), which underscore the relation between animal well-being, public health, and food quality. However, these results contrast with earlier studies. Vimiso *et al*. (2012) found that consumers and traders prioritize price, color, and fat content of the meat as main factors, without linking them directly to animal well-being practices. This disparity suggests a gap in the perception of the consumer regarding factors that determine meat quality.

Cluster analysis

Four clusters were formed (Figure 2), which describe the differences found in the perceptions of slaughterers in the barbacoa production chain.

Cluster 1

This was composed of the largest number of barbacoa producers (20), with a middle-high school education level, on average. This cluster stood out for having differences

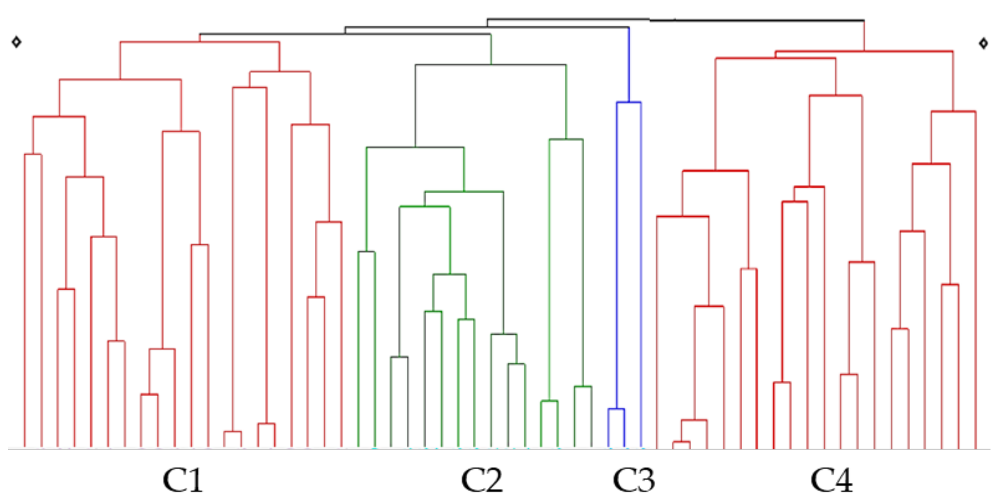

Figure 2. Hierarchical clusters (dendrogram) of the analysis on perceptions of slaughterers and barbacoa producers from Capulhuac de Mirafuentes, State of Mexico.

($p < 0.05$) with the other clusters. This group claims that slaughterhouses are probably not an important element in the lamb production chain. In their vision, which they share with cluster 3, there is undoubtedly no impact of the methods and/or ways of slaughtering on the characteristics of the lamb. Likewise, they consider that animals that suffer do not generate desirable meat. Likewise, they agree with cluster 2 that there is surely not enough information on the well-being of farm animals in Mexico.

Cluster 2

This was composed of 15 barbacoa producers with an elementary-middle school education, who displayed significant differences in perception (p £ 0.05) in comparison to the other clusters. For this group, the importance of the well-being of farm animals was the lowest among clusters. It also featured the lowest level of knowledge on the living conditions of farm animals. Additionally, it coincides with cluster 1 that there surely is not enough information on the well-being of farm animals in Mexico.

Cluster 3

Composed of three barbacoa producers with an elementary-middle school education, this cluster displays significant differences (p £ 0.05) with the other groups, mainly regarding not considering that farm animals feel pain, although they are aware that they probably have emotions. Likewise, they consider that stress produced in animals during production and transportation probably does not affect the quality of the meat. On the other hand, they agree with cluster 2 that new laws are required regarding the well-being of animals to avoid the abuse of animals in slaughterhouses.

Cluster 4

Composed of 15 barbacoa producers with elementary-middle school education. They display significant differences (p £ 0.05) with other clusters in their belief that slaughterhouses probably do not manage animals correctly during pre-slaughter and slaughtering. This cluster probably does not believe that slaughterhouses comply with adequate regulations to slaughter animals (p £ 0.05).

The cluster analysis showed that most participants were rural men with a medium education level (middle or high school). This characteristic is relevant, as mentioned by Ashuro *et al*. (2023), who showed that handlers with higher education levels displayed significantly better personal hygiene practices and a greater willingness to participate in training programs. On the other hand, Tegegne (2017) found a strong correlation between education and knowledge on food security, as well as with the washing of hands. Gil *et al*. (2024) points out that the hygienic handling of meat is related to the level of education of the operators, so long as there is constant supervision and training, with the latter two practices being relevant for slaughterers and barbacoa producers, who believe that they are not being implemented in Capulhuac de Mirafuentes (Table 3).

Table 3. Key characteristics identified in the clusters formed by the perception analysis of slaughterers and barbacoa producers in Capulhuac de Mirafuentes, State of Mexico.

Study variable Respondents	Cluster 1 20	Cluster 2 15	Cluster 3 3	Cluster 4 15	$p \leq 0.05$
Do you believe sheep slaughterhouses are important for the municipality of Capulhuac de Mirafuentes?	5	5	5	5	0.092
Do you believe lamb is important for the municipality of Capulhuac de Mirafuentes?	5	5	5	5	0.088
Do you believe slaughterhouses to be an important link in the sheep production chain?	2	5	5	5	0.001
Do you believe the methods and/or ways of slaughter affect the properties of the lamb?	1	5	1	5	0.001
Do you believe that animals that suffer generate less desirable meat?	1	5	1	5	0.001
Do you believe the meat from animals raised in complete freedom is better?	5	5	5	5	0.365
Do you believe slaughterhouses comply with adequate regulations for slaughtering animals?	5	5	1	2	0.015
Do you believe slaughterhouses handle animals correctly during pre-slaughtering?	5	5	4	2	0.001
Do you believe slaughterhouses handle animals correctly during slaughtering?	5	5	4	2	0.001
Do you believe animals experience feelings of pain, anguish, and fear before being taken to be slaughtered?	5	5	5	5	0.45
On a scale of 1 to 10, how important is the well-being of farm animals to you?	8	8	8	10	0.004
Do you believe slaughterhouse operators and workers should be educated on animal well-being?	5	5	5	5	0.8
Do you believe new laws are needed regarding animal well-being to avoid the abuse of animals in slaughterhouses?	5	4	4	5	0.006
What is your level of knowledge on the living standards of farm animals?	Medium	Low	High	Low-medium	0.012
Should farm animals be well-fed, warm, and healthy?	5	5	5	5	0.131
Should farm animals be able to express the natural behaviors of their species?	5	5	5	5	0.451
Should farm animals be free from fear and stress?	5	5	5	5	0.138
Do farm animals feel pain?	5	5	2	5	0.005
Are farm animals able to feel emotions (positive or negative)?	5	5	4	5	0.041
Do you believe there is enough information in Mexico on the well-being of farm animals?	1	1	2	2	0.037
Do you believe the well-being and the protection of farm animals in Mexico should be improved?	5	5	5	5	0.767
Do you believe stress in animals during production and transportation can affect the quality of the meat consumed?	5	5	2	5	0.002

1: definitely not; 2: probably not; 3: indifferent; 4: probably so; 5: definitely so.

In general terms, survey respondents consider the slaughtering of sheep and the marketing of meat and barbacoa as imperative economic activities for the municipality of Capulhuac de Mirafuentes, and they are aware that farm animals should be well fed, warm, and free from fear or anguish. However, they do not understand the negative impact that omitting such conditions may have on meat safety and quality. This is one of the main consequences related to the appearance of the dark, firm, and dry meat (DFD) syndrome, which reduces tenderness, sensorial acceptability, and yield of the carcass, with a direct influence on the acceptance of consumers (Koscinczuk, 2014; Quiroz-Osorio *et al*., 2016; Hernández-Hernández *et al*., 2023).
Several authors point out that muscle undergoes physical and biochemical changes due to a reduction in blood flow, which interrupts oxygen uptake by myoglobin to the mitochondria. This process triggers a series of enzyme adaptations that try to obtain energy from the stored glycogen (Zhang *et al*., 2019) through the metabolic pathway of anaerobic glycolysis. This produces small amounts of ATP, carbon dioxide, and lactic acid (Zhao *et al*., 2022), which produce pH levels higher than 5.6 *post-mortem* in the carcass, with direct repercussions on the sensory characteristics perceived by consumers (meat texture, color, taste, and water-retaining ability) (Terlouw and Gagaoua, 2023). As a result, it is critical to implement humane and standardized procedures that reduce animal suffering while ensuring safe and high-quality meat.

CONCLUSIONS

Slaughterers and barbacoa producers in the municipality of Capulhuac de Mirafuentes identify the lack of education as the main problem in topics related to animal well-being. This aspect is essential to guarantee that animals are raised under conditions that allow them to express the natural behaviors of their species. Additionally, it ensures that their nutritional and physical health needs are met and that stress is minimized throughout the whole production process. These factors are acknowledged to have a direct impact on the sensory properties of meat, such as taste, texture, and overall quality. For this reason, training programs focusing on adequate livestock production and animal well-being, as well as slaughtering and food safety, must be implemented to contribute to improving productive practices in municipal slaughterhouses. These actions guarantee a safe and high-quality end product for the consumer.

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Agrociencia

APPLICATION OF LAGRANGE MULTIPLIERS IN TWO-WAY CLASSIFICATION ANALYSIS OF VARIANCE WITH INTERACTION

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ABSTRACT

The objective of this study was to use Lagrange multipliers to obtain the solution to the normal equations, followed by the sums of squares of the analysis of variance, for a two-way classification model with interaction. The model included the main effects of rations and sires at two and three levels, respectively, as well as their interaction. The least squares method was used in an unbalanced data set with all cells filled. The modified normal equations were formed with the matrix $(\boldsymbol{X'X})_{(12,12)}$ in the upper part, together with the matrix $\boldsymbol{P}_{(12x6)}$ in the lower part, its transpose $\boldsymbol{P}'_{(6x12)}$ appears along with the zero matrix $\mathbf{0}_{(6x6)}$, which together constitutes the left-hand side (LHS) of the modified normal equations. Both the parameter vector and the right-hand side (RHS) were augmented with six θ's and six 0's, respectively. In the inverse of the LHS of the modified equations, within the section corresponding to $(\boldsymbol{X'X})$, the inverse of the coefficient matrix of the incomplete rank matrix was obtained, which resulted in the same property. It was verified, however, that this inverse is the generalized inverse corresponding to the system of equations when the sum of the constants is assumed to be zero for each effect. The direct procedure was used for the decomposition of the models' sum of squares, where both the vector of parameter estimators and the inverse elements of the submatrix for each effect were reduced according to their corresponding degrees of freedom. The results were identical to those previously obtained with conventional procedures, with the advantage that all inverse elements were available for calculating standard errors, reducing the possibility of errors.

Keywords: Confounding effect, orthogonality, least squares, incomplete rank.

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INTRODUCTION

Lack of orthogonality in biological experiments can occur from deficiencies in the experimental material that prevent all treatments to be assigned to their respective blocks. This forces the researcher to use designs such as balanced incomplete blocks or partially balanced incomplete blocks. Such imbalance is referred to as planned unbalance (Searle, 1987), in which treatments are compared with equal variances, or treatment groups with similar variances, respectively (Martin, 1995). However, lack of orthogonality often occurs as a result of accidents during the experimental phase, such as deaths of experimental units or measurement errors that require deleting observations from the experiment. From a statistical perspective, these issues lead to severe problems in the computation of sums of squares. This situation is known as unplanned lack of orthogonality and results in consequences such as fewer degrees of freedom than expected for the source of variation or, in extreme cases, a sum of squares equal to zero for that factor in the analysis of variance (Searle, 1987; Martin, 1995).

One of the consequences of imbalance is that, in experiments with two or more factors, confounding effects occur because a correlation is established between the effects. This leads to difficulties when performing hypothesis tests, since linear functions of the parameter estimates are contaminated by fractions of other factors, requiring the assumption that these confounding effects are zero in order for the tested hypothesis to be valid (Steel *et al.,* 1997; Littell *et al.,* 2010).

There are several alternatives to obtain the solution to a system of linear equations. The simplest is to augment the coefficient matrix with the right-hand side of the normal equations and to perform row operations until reaching the reduced row echelon form. However, the inverse elements of the coefficient matrix will still be required as part of the statistical inference process. To solve the normal equations, the most common alternatives are either to set some elements to zero and to use a generalized inverse or to assume that the sum of the constants for each factor is zero (Harvey, 1960; Damond and Harvey, 1987). Given the invariance property of certain quantities typical of the analysis of variance, either of these two procedures is sufficient (Searle, 1971).

The use of Lagrange multipliers is described in detail from a theoretical standpoint and illustrated with a numerical example for the one-way classification analysis (Searle, 1971; 1987). Henderson (1978) the undesirable properties of regressed least squares for predicting animal genetic merit using linear mixed models. However, in this case, to address the linear dependence problem on the left-hand side of the normal equations (LHM), the author simplified the calculations by dropping the mean equation, thereby reducing the problem to a single linear dependence in the system of equations, which is a strategy consistently used by Searle (1971) in solving least squares estimation problems. The literature on the use of Lagrange multipliers in the context of linear models is not very abundant, it was later addressed by Zhu and Li (2007) and more recently in large-scale problems by Scott J, and Tůma M (2022).

Due to the above, the present research has the objective to implement Lagrange multipliers to solve the problem of linear dependence in the LHM of the coefficient matrix of the normal equations in a two-way classification model with interaction. In addition, it aims to obtain all the inverse elements in order to avoid errors that may arise when calculating them from the dependency relationships among the effects in the model.

MATERIALS AND METHODS

For this research, data from an example published by Harvey (1960) are used to analyze a two-way classification experiment with two factors: rations at two levels and sires at three levels, including their interaction, with all cells filled but an unequal number of observations in each (Table 1).

Table 1. Data used to illustrate a two-way classification model with interaction between rations and sires. Adapted from Harvey (1960).

		Sire (*j*)					
Ration (*i*)		1	2	3	$y_{i...}$	$n_{i...}$	
1	y_{1jk}	5–6	2-3-5-6-7	3	37	8	
	$y_{1j.}$	11	23	3			
	$n_{1j.}$	2	5	1			
2	y_{2jk}	2–3	8–8-9	4–4–6–6-7	61	10	
	$y_{2j.}$	5	25	31			
	$n_{2j.}$	2	3	5			
	$y_{.j.}$	16	48	34			
	$n_{.j.}$	4	8	6		$y_{...}$	94
						$n_{...}$	18

i, *j*, *k* = subscript for ration, sire and individual observation identification respectively; y_{1jk} = individual observations for ration 1 and sire j; $y_{1j.}$ = sum of observations in ration 1 for sires 1, 2 and 3; $n_{1j.}$ = number of observations in ration 1 for sires 1, 2 and 3; y_{2jk} = individual observations in ration 2 and sire j; $y_{2j.}$ = sum of observations on ration2 for sires 1, 2 and 3; $n_{2j.}$ = number of observations for sires 1,2 and 3 in ration 2; $y_{.j.}$ = sum of observations for sire *j*; $n_{.j.}$ = number of observations for sire *j*; $y_{i...}$ = sum of observation for ration *i*; $n_{i...}$ = number of observation for sire *i*; $y_{...}$ = grand total and $n_{...}$ = total observations in the data set.

The model, expressed using matrix algebra, can be written as:

$$\boldsymbol{y} = \boldsymbol{Xb} + \boldsymbol{e} \quad (1)$$

where y is the observation vector, and $\boldsymbol{X}$ is an incidence matrix consisting of zeros and ones, which relates elements of the observation vector to the effects of the factors included in the model. In this case, the twelve columns of are represented as follows: one for the mean, μ; two for the ration effects, α_i, i=1, … ,2; three for the sire effects, β_j, j=1, … 3; and six for the interaction between the them, $(\alpha\beta)_{i,j}$; the number of observations within each cell, varied from k=1, … , $n_{..k}$. The vector $\boldsymbol{b}$ contains the parameters to be estimated by least squares, assuming that the factors included in the model are fixed, with distributional properties as follows: mean, $E(\boldsymbol{y}) = \boldsymbol{Xb}$ and variance $V(\boldsymbol{y}) = \boldsymbol{I}\sigma^2$; finally, $\boldsymbol{e}$ represents the errors with distributional properties $E(\boldsymbol{e}) = \boldsymbol{0}$ and $V(\boldsymbol{e}) = \boldsymbol{I}\sigma^2$, which are assumed to be independent of each other, so that $Cov\ (e_{ijk}, e_{ijk'}) = 0$. The matrix representation of model (1) is shown in terms of the data used in Table 2.

$$\begin{bmatrix} 1&1&0&1&0&0&1&0&0&0&0&0 \\ 1&1&0&1&0&0&1&0&0&0&0&0 \\ 1&1&0&0&1&0&0&1&0&0&0&0 \\ 1&1&0&0&1&0&0&1&0&0&0&0 \\ 1&1&0&0&1&0&0&1&0&0&0&0 \\ 1&1&0&0&1&0&0&1&0&0&0&0 \\ 1&1&0&0&1&0&0&1&0&0&0&0 \\ 1&1&0&0&0&1&0&0&1&0&0&0 \\ 1&0&1&1&0&0&0&0&0&1&0&0 \\ 1&0&1&1&0&0&0&0&0&1&0&0 \\ 1&0&1&0&1&0&0&0&0&1&0&0 \\ 1&0&1&0&1&0&0&0&0&0&1&0 \\ 1&0&1&0&1&0&0&0&0&0&1&0 \\ 1&0&1&0&0&1&0&0&0&0&0&1 \\ 1&0&1&0&0&1&0&0&0&0&0&1 \\ 1&0&1&0&0&1&0&0&0&0&0&1 \\ 1&0&1&0&0&1&0&0&0&0&0&1 \\ 1&0&1&0&0&1&0&0&0&0&0&1 \end{bmatrix} \begin{bmatrix} \hat{\mu} \\ \hat{\alpha}_1 \\ \hat{\alpha}_2 \\ \hat{\beta}_1 \\ \hat{\beta}_2 \\ \hat{\beta}_3 \\ (\widehat{\alpha\beta})_{11} \\ (\widehat{\alpha\beta})_{12} \\ (\widehat{\alpha\beta})_{13} \\ (\widehat{\alpha\beta})_{21} \\ (\widehat{\alpha\beta})_{22} \\ (\widehat{\alpha\beta})_{23} \end{bmatrix} = \begin{bmatrix} 5 \\ 6 \\ 2 \\ 3 \\ 5 \\ 6 \\ 7 \\ 3 \\ 2 \\ 3 \\ 8 \\ 8 \\ 9 \\ 4 \\ 4 \\ 6 \\ 6 \\ 7 \end{bmatrix}$$

Table 2. Equation of the two-way classification model with interaction model.

Searle (1971) proposed implementing restrictions on the model, indicating that the least squares solution leads to solving the next system of equations:

$$\begin{bmatrix} \boldsymbol{X'X} & \boldsymbol{P} \\ \boldsymbol{P'} & \boldsymbol{0} \end{bmatrix} \begin{bmatrix} \boldsymbol{\beta^o} \\ \boldsymbol{\theta} \end{bmatrix} = \begin{bmatrix} \boldsymbol{X'y} \\ \boldsymbol{0} \end{bmatrix} \tag{2}$$

In an ordinary least squares problem, $\boldsymbol{X'X}$ represents the coefficient matrix for the normal equations. In this case, it is an incomplete rank matrix whose weakness is to be addressed by applying this methodology. $\boldsymbol{P}$ is a matrix required to be of full column rank in order to solve the linear dependence problem present in $\boldsymbol{X'X}$. $\boldsymbol{P'}$ is the transpose of $\boldsymbol{P}$; and $\boldsymbol{0}$ represents a square matrix of zeros, with dimensions sufficient to maintain the symmetry of the modified coefficient matrix used in the methodology. Together, these matrices form the LHM. The parameter vector must be augmented with a column subvector $\boldsymbol{\theta}$ containing as many elements as there are dependencies. On the right-hand side of the normal equations (RHM), the original $\boldsymbol{X'y}$ vector is arranged with the totals and subtotals corresponding to the mean and the remaining factor levels, augmented with as many zeros as the number of dependencies in the problem.

The sums of squares for each source of variation are obtained using the direct procedure by multiplying the transpose of the reduced segment for each factor of the solution vector by the corresponding inverse of the reduced segment of the inverse by the reduced segment of the vector (Harvey, 1960) All calculations were performed using MATLAB version 8.5.0 (R2015a) (The MathWorks Inc., 2015) on an ACER Aspire 3 computer, model N20C5, equipped with 20 GB of random-access memory.

RESULTS AND DISCUSSION

In general terms, for this specific problem, the solution using the least squares method combined with the principle of Lagrange multipliers is straightforward. The procedure involved computing the matrix product $\boldsymbol{X'X}$, which in this case is a 12 × 12 matrix of rank six, and identifying the six dependency relationships in the coefficient matrix. A matrix $\boldsymbol{P}$ is defined to impose restrictions ensuring that the sum of the effects within each factor is zero, with its transpose $\boldsymbol{P'}$ used to maintain symmetry. A 6 × 6 matrix of zeros is included in the modified normal equations, and the parameter vector and RHM are augmented with six θ's and six $0'$s, respectively. The rest of the analysis follows the standard least squares method using matrix algebra. This approach is consistent with the procedures outlined by Searle (1971) and Henderson (1978).

The normal equations and the inverse of the coefficient matrix

The normal equations applying the principle of Lagrange multipliers for this problem according to (2), are expressed as follows:

$$
\left[\begin{array}{cccccccccccc|cccccc}
18 & 8 & 10 & 4 & 8 & 6 & 2 & 5 & 1 & 2 & 3 & 5 & 0 & 0 & 0 & 0 & 0 & 0 \\
8 & 8 & 0 & 2 & 5 & 1 & 2 & 5 & 1 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 \\
10 & 0 & 10 & 2 & 3 & 5 & 0 & 0 & 0 & 2 & 3 & 5 & 1 & 0 & 0 & 0 & 0 & 0 \\
4 & 2 & 2 & 4 & 0 & 0 & 2 & 0 & 0 & 2 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 \\
8 & 5 & 3 & 0 & 8 & 0 & 0 & 5 & 0 & 0 & 3 & 0 & 0 & 1 & 0 & 0 & 0 & 0 \\
6 & 1 & 5 & 0 & 0 & 6 & 0 & 0 & 1 & 0 & 0 & 5 & 0 & 1 & 0 & 0 & 0 & 0 \\
2 & 2 & 0 & 2 & 0 & 0 & 2 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 & 1 & 0 \\
5 & 5 & 0 & 0 & 5 & 0 & 0 & 5 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 & 1 \\
1 & 1 & 0 & 0 & 0 & 1 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 \\
2 & 0 & 2 & 2 & 0 & 0 & 0 & 0 & 0 & 2 & 0 & 0 & 0 & 0 & 0 & 1 & 1 & 0 \\
3 & 0 & 3 & 0 & 3 & 0 & 0 & 0 & 0 & 0 & 3 & 0 & 0 & 0 & 0 & 1 & 0 & 1 \\
5 & 0 & 5 & 0 & 0 & 5 & 0 & 0 & 0 & 0 & 0 & 5 & 0 & 0 & 0 & 1 & 0 & 0 \\
\hline
0 & 1 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 1 & 1 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & 0 & 1 & 1 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 1 & 1 & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0
\end{array}\right]
\begin{bmatrix} \mu \\ \alpha_1 \\ \alpha_2 \\ \beta_1 \\ \beta_2 \\ \beta_3 \\ (\alpha\beta)_{11} \\ (\alpha\beta)_{12} \\ (\alpha\beta)_{13} \\ (\alpha\beta)_{21} \\ (\alpha\beta)_{22} \\ (\alpha\beta)_{23} \\ \cdots \\ \theta_1 \\ \theta_2 \\ \theta_3 \\ \theta_4 \\ \theta_5 \\ \theta_6 \end{bmatrix}
=
\begin{bmatrix} 94 \\ 37 \\ 57 \\ 16 \\ 48 \\ 30 \\ 11 \\ 23 \\ 3 \\ 5 \\ 25 \\ 27 \\ \cdots \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \end{bmatrix}
\qquad (3)
$$

The LHM is labeled as the coefficient matrix of the system $\boldsymbol{M}_{18x18}$, represented in a partitioned form according to Searle (1971). The elements of the original incomplete rank matrix are assigned to $\boldsymbol{M}_{(1:12,\,1:12)}$ as the $(\boldsymbol{X}'\boldsymbol{X})_{12x12}$. The submatrix $\boldsymbol{M}_{(1:12,\,18:18)}$ corresponds to the full column rank matrix $P_{(12x6)}$, with its transpose $\boldsymbol{P}'_{(6x12)}$ in the submatrix $\boldsymbol{M}_{(18:18,\,1:12)}$. A zero matrix $\mathbf{0}_{6x6}$ is defined in positions $\boldsymbol{M}_{(13:19,\,13:18)}$. The parameter vector is augmented with a subvector of six θ_1 restrictions corresponding to the dependencies, and the RHS vector of the normal equations is similarly augmented with six zeros.

Following the matrix of the normal equations in (3) and $\boldsymbol{P}'_{(6x12)}$, the restrictions imposed on the normal equations to bring $\boldsymbol{M}_{18x18}$ to full rank can be deduced. The first $\boldsymbol{\theta}_1' = [0|1\ 1|0]_{(1,12)}$, allows to assume that the sum of both rations is zero, $\alpha_1 + \alpha_2 = 0$. The second, $\boldsymbol{\theta}_2' = [0|1\ 1\ 1|0]_{(1,12)}$, ensures that the sire effects sum to zero. For the interaction, four θ_i restrictions are required: $\boldsymbol{\theta}_3$ sets the sire subcells to sum to zero at the first level of rations; $\boldsymbol{\theta}_4$ does the same at the second level of rations; $\boldsymbol{\theta}_5$ sets the ration subcells to sum to zero at the first sire level; and $\boldsymbol{\theta}_6$ applies the same procedure at the second sire level. By structuring the coefficient matrix in this manner, the matrix $\boldsymbol{M}_{18x18}$ attains linearly independent columns, ensuring the existence of an inverse and a unique solution to the system of equations (Table 3).

$$\left[\begin{array}{cccccccccccc:cccccc}
-0.0759 & 0.0185 & -0.0185 & 0.0074 & -0.0315 & 0.0241 & -0.0185 & -0.0296 & 0.0481 & 0.0185 & 0.0296 & -0.0481 & -0.5 & -0.3333 & -0.1667 & -0.1667 & 0. & 0. \\
0.0185 & 0.0759 & -0.0759 & -0.0185 & -0.0296 & 0.0481 & 0.0074 & -0.0315 & 0.0241 & -0.0074 & 0.0315 & -0.0241 & 0.5 & 0. & -0.1667 & 0.1667 & 0. & 0. \\
-0.0185 & -0.0759 & 0.0759 & 0.0185 & 0.0296 & -0.0481 & -0.0074 & 0.0315 & -0.0241 & 0.0074 & -0.0315 & 0.0241 & 0.5 & 0. & 0.1667 & -0.1667 & 0. & 0. \\
0.0074 & -0.0185 & 0.0185 & 0.1593 & -0.0519 & -0.1074 & 0.0185 & 0.0296 & -0.0481 & -0.0185 & -0.0296 & 0.0481 & 0. & 0.3333 & 0.1667 & 0.1667 & -0.5 & 0. \\
-0.0315 & -0.0296 & 0.0296 & -0.0519 & 0.1204 & -0.0685 & 0.0296 & 0.0074 & -0.0370 & -0.0296 & -0.0074 & 0.0370 & 0. & 0.3333 & 0.1667 & 0.1667 & 0. & -0.5 \\
0.0241 & 0.0481 & -0.0481 & -0.1074 & -0.0685 & 0.1759 & -0.0481 & -0.0370 & 0.0852 & 0.0481 & 0.0370 & -0.0852 & 0. & 0.3333 & -0.3333 & -0.3333 & 0.5 & 0.5 \\
-0.0185 & 0.0074 & -0.0074 & 0.0185 & 0.0296 & -0.0481 & 0.1593 & -0.0519 & -0.1074 & -0.1593 & 0.0519 & 0.1074 & 0. & 0. & 0.1667 & -0.1667 & 0.5 & 0. \\
-0.0296 & -0.0315 & 0.0315 & 0.0296 & 0.0074 & -0.0370 & -0.0519 & 0.1204 & -0.0685 & 0.0519 & -0.1204 & 0.0685 & 0. & 0. & 0.1667 & -0.1667 & 0. & 0.5 \\
0.0481 & 0.0241 & -0.0241 & -0.0481 & -0.0370 & 0.0852 & -0.1074 & -0.0685 & 0.1759 & 0.1054 & 0.0685 & -0.1759 & 0. & 0. & 0.6667 & 0.3333 & -0.5 & -0.5 \\
0.0185 & -0.0074 & 0.0074 & -0.0181 & -0.0296 & 0.0481 & -0.1593 & 0.0519 & 0.1074 & 0.1595 & -0.0519 & -0.1074 & 0. & 0. & -0.1667 & 0.1667 & 0.5 & 0. \\
0.0296 & 0.0315 & -0.0315 & -0.0296 & -0.0074 & 0.0370 & 0.0519 & -0.1204 & 0.0685 & -0.0519 & 0.1204 & -0.0685 & 0. & 0. & -0.1667 & 0.1667 & 0. & 0.5 \\
-0.0481 & -0.0241 & 0.0241 & 0.0481 & 0.0370 & -0.0852 & 0.1074 & 0.0685 & -0.1759 & -0.1074 & -0.0685 & 0.1759 & 0. & 0. & 0.3333 & 0.6667 & -0.5 & -0.5 \\
\cdots & \cdots & \cdots & \cdots & \cdots & \cdots & \cdots & \cdots & \cdots & \cdots & \cdots & \cdots & \cdots & \cdots & \cdots & \cdots & \cdots & \cdots \\
-0.5 & 0.5 & 0.5 & 0. & 0. & 0. & 0. & 0. & 0. & 0. & 0. & 0. & 0. & 0. & 0. & 0. & 0. & 0. \\
-0.3333 & 0. & 0. & 0.3333 & 0.3333 & 0.3333 & 0. & 0. & 0. & 0. & 0. & 0. & 0. & 0. & 0. & 0. & 0. & 0. \\
-0.1667 & -0.1667 & 0.1667 & 0.1667 & 0.1667 & -0.3333 & 0.1667 & 0.1667 & 0.6667 & -0.1667 & -0.1687 & 0.3333 & 0. & 0. & 0. & 0. & 0. & 0. \\
-0.1667 & 0.1667 & -0.1667 & 0.1667 & 0.1667 & -0.3333 & -0.1667 & -0.1667 & 0.3333 & 0.1667 & 0.1687 & 0.6667 & 0. & 0. & 0. & 0. & 0. & 0. \\
0. & 0. & 0. & -0.5000 & 0. & 0.5 & 0.5 & 0. & -0.5 & 0.5 & 0. & -0.5 & 0. & 0. & 0. & 0. & 0. & 0. \\
0. & 0. & 0. & 0. & -0.5000 & 0.5 & 0. & 0.5 & -0.5 & 0. & 0.5 & -0.5 & 0. & 0. & 0. & 0. & 0. & 0.
\end{array}\right]$$

Table 3. Inverse of the coefficient matrix M of the normal equations, augmented according to the Lagrange multipliers methodology and partitioned according to the elements of the original matrix.

The matrix $\boldsymbol{G}_{12x12}$, which contains all the inverse elements corresponding to the incomplete rank matrix $\boldsymbol{X}'\boldsymbol{X}$, has been partitioned by rows and columns according to the model terms for clarity. These partitions correspond to the mean, the rations, the sire, and their interaction effects:

$$\boldsymbol{G} = \left[\begin{array}{cccccccccccccccc}
0.0759 & \vdots & 0.0185 & -0.0185 & \vdots & 0.0074 & -0.0315 & 0.0241 & \vdots & -0.0185 & -0.0296 & 0.0481 & 0.0185 & 0.0296 & -0.0481 \\
\cdots & & \cdots & \cdots & & \cdots & \cdots & \cdots & & \cdots & \cdots & \cdots & \cdots & \cdots & \cdots \\
0.0185 & \vdots & 0.0759 & -0.0759 & \vdots & -0.0185 & -0.0296 & 0.0481 & \vdots & 0.0074 & -0.0315 & 0.0241 & -0.0074 & 0.0315 & -0.0241 \\
-0.0185 & \vdots & -0.0759 & 0.0759 & \vdots & 0.0185 & 0.0296 & -0.0481 & \vdots & -0.0074 & 0.0315 & -0.0241 & 0.0074 & -0.0315 & 0.0241 \\
\cdots & & \cdots & \cdots & & \cdots & \cdots & \cdots & & \cdots & \cdots & \cdots & \cdots & \cdots & \cdots \\
0.0074 & \vdots & -0.0185 & 0.0185 & \vdots & 0.1593 & -0.0519 & -0.1074 & \vdots & 0.0185 & 0.0296 & -0.0481 & -0.0185 & -0.0296 & 0.0481 \\
-0.0315 & \vdots & -0.0296 & 0.0296 & \vdots & -0.0519 & 0.1204 & -0.0685 & \vdots & 0.0296 & 0.0074 & -0.0370 & -0.0296 & -0.0074 & 0.0370 \\
0.0241 & \vdots & 0.0481 & -0.0481 & \vdots & -0.1074 & -0.0685 & 0.1759 & \vdots & -0.0481 & -0.0370 & 0.0852 & 0.0481 & 0.0370 & -0.0852 \\
\cdots & & \cdots & \cdots & & \cdots & \cdots & \cdots & & \cdots & \cdots & \cdots & \cdots & \cdots & \cdots \\
-0.0185 & \vdots & 0.0074 & -0.0074 & \vdots & 0.0185 & 0.0296 & -0.0481 & \vdots & 0.1593 & -0.0519 & -0.1074 & -0.1593 & 0.0519 & 0.1074 \\
-0.0296 & \vdots & -0.0315 & 0.0315 & \vdots & 0.0296 & 0.0074 & -0.0370 & \vdots & -0.0519 & 0.1204 & -0.0685 & 0.0519 & -0.1204 & 0.0685 \\
0.0481 & \vdots & 0.0241 & -0.0241 & \vdots & -0.0481 & -0.0370 & 0.0852 & \vdots & -0.1074 & -0.0685 & 0.1759 & 0.1074 & 0.0685 & -0.1759 \\
0.0185 & \vdots & -0.0074 & 0.0074 & \vdots & -0.0185 & -0.0296 & 0.0481 & \vdots & -0.1593 & 0.0519 & 0.1074 & 0.1593 & -0.0519 & -0.1074 \\
0.0296 & \vdots & 0.0315 & -0.0315 & \vdots & -0.0296 & -0.0074 & 0.0370 & \vdots & 0.0519 & -0.1204 & 0.0685 & -0.0519 & 0.1204 & -0.0685 \\
-0.0481 & \vdots & -0.0241 & 0.0241 & \vdots & 0.0481 & 0.0370 & -0.0852 & \vdots & 0.1074 & 0.0685 & -0.1759 & -0.1074 & -0.0685 & 0.1759
\end{array}\right] \tag{4}$$

The submatrix $\boldsymbol{G}_{2,3:\,2,3}$ represents the inverse the augmented ration effect; the element $\boldsymbol{G}_{(2,3)}$ is the negative of $\boldsymbol{G}_{(2,2)}$, so that the rows and columns sum to zero, thus satisfying the imposed restriction. The same can be observed for the sire effects in the submatrix $\boldsymbol{G}_{(4,6:\,4,6)}$: the negative of the sum of (0.1593 - 0.0519) is -0.1074, and the negative of the sum of (-0.0519 + 0.1204) is -0.0685, which also occurs by columns within this submatrix. The same can be demonstrated in the submatrix $\boldsymbol{G}_{(7,12:\,7,12)}$, corresponding to the interaction effects. The inverse elements corresponding to this submatrix have the same magnitude as those published by Harvey (1960), with differences in the positions of the elements. This author did not show the values of the inverse elements for the rows and columns eliminated to break the linear dependence in the coefficient matrix, although he illustrated the calculations.

With ($\boldsymbol{X}'\boldsymbol{X}$) being the incomplete-rank coefficient matrix of the original normal equations, it can be verified using linear algebra software that G is a generalized inverse, since it satisfies the properties ($\boldsymbol{X}'\boldsymbol{X}$) $\boldsymbol{G}$ ($\boldsymbol{X}'\boldsymbol{X}$) = ($\boldsymbol{X}'\boldsymbol{X}$) and $\boldsymbol{G}$ ($\boldsymbol{X}'\boldsymbol{X}$) $\boldsymbol{G}$ = $\boldsymbol{G}$. This generalized inverse of $X'X$ corresponds to the generalized inverse when imposed the constrain that the sum of the constants within each effect add up to zero. However, it fails to comply with the symmetry properties required by a Moore-Penrose inverse, as pointed out by Searle (1982), specifically in points (iii) and (iv) corresponding to the products ($\boldsymbol{X}'\boldsymbol{X}$) $\boldsymbol{G}$ and $\boldsymbol{G}$ ($\boldsymbol{X}'\boldsymbol{X}$), respectively.

$$\boldsymbol{\beta}^{0\prime} = [4.8889 - 0.5222\ 0.5222 - 0.8889\ 1.5778 - 0.6889\ 2.0222 \\ - 1.3444 - 0.6778 - 2.0222 \\ - 1.3444\ 0.67780 \quad 0 \quad 0 \quad 0 \quad 0 \quad 0]$$

The solution vector to the normal equations is:

The elements corresponding to the estimators of the model parameters are identical to those previously published, with differences only in their arrangement (Harvey, 1960), as that author presented the results placing the factors in the order of sires, rations, and sires × rations.

Computing the sum of squares for the analysis of variance

If $\boldsymbol{y}$, $\mathbf{1'}_{12}$, $\boldsymbol{\beta'}_0$, and $\boldsymbol{X'y}$ denote the observation vector, the summing vector, the parameter estimator vector, and the RHM, respectively, the sums of squares are obtained in the usual manner. The reduction in the sum of squares due to the mean, often called the correction factor, is: $SC_\mu = [(\mathbf{1'}_{12y})\ (\mathbf{1'}_{12y})]\ /\ (\mathbf{1'}_{12}\mathbf{1'}_{12}) = 490.8889$. The total sum squares corrected for the mean is $SC_{Total|\mu} = \boldsymbol{y'y} - SC_\mu = 77.1111$. The reduction in the sum squares due to the full model is $SS_{R(\mu,\ \alpha_i,\ \beta_j,\ (\alpha\beta)_{ij})} = \boldsymbol{\beta'}_0\ \boldsymbol{X'y} = 541.9333$, and finally, the error sum of squares is estimated as $SC_{error} = \boldsymbol{y'y} - \boldsymbol{\beta'}_0\ \boldsymbol{X'y} = 26.0667$.

Decomposition of the sum of squares for the full model

There are several alternatives for obtaining the sums of squares for the main effects and the interaction. In this study, however, the direct procedure described by Harvey (1960) is applied. It should be noted that, in this case, both the matrix $\boldsymbol{G}$ and the submatrices corresponding to the factors included in the model are of incomplete rank, due to the restrictions imposed. To obtain the sums of squares using the direct procedure, each submatrix had to be reduced to full rank by eliminating the linearly dependent rows and columns. The same reduction is applied to the vector of parameter estimators, with the corresponding subvectors adjusted accordingly. The sums of squares for the effects are computed as follows:

Sum of squares for the ration effects

$$SC_{ration} = \hat{\alpha}_1'\ (\boldsymbol{G}_R'\boldsymbol{G}_R)^{-1}\hat{\alpha}_1$$
$$= (-0.5222)(0.0759)^{-1}(-0.5222)$$

$SC_{ration} = 3.5919$

Sum of squares for the sire effects

$$SC_{sire} = [\hat{\beta}_1 \quad \hat{\beta}_2]'\ (\boldsymbol{G}_P'\boldsymbol{G}_P)^{-1}\begin{bmatrix}\hat{\beta}_1\\ \hat{\beta}_2\end{bmatrix}$$
$$= [-0.8889 \quad 1.5778]'\begin{bmatrix}0.1593 & -0.0519\\ -0.0519 & 0.1204\end{bmatrix}^{-1}\begin{bmatrix}-0.8889\\ 1.5778\end{bmatrix}$$

$SC_{sire} = 21.0009$

Sum of squares for the sire× ration interaction effects

$$SC_{ration*sire} = \left[\left(\widehat{\alpha\beta}\right)_{11} \quad \left(\widehat{\alpha\beta}\right)_{12}\right]'\ (\boldsymbol{G}_{rs}'\boldsymbol{G}_{rs})^{-1}\begin{bmatrix}\left(\widehat{\alpha\beta}\right)_{11}\\ \left(\widehat{\alpha\beta}\right)_{12}\end{bmatrix}$$
$$= [2.0222 \quad -1.3444]'\begin{bmatrix}0.1593 & -0.0519\\ -0.0519 & 0.1204\end{bmatrix}^{-1}\begin{bmatrix}2.0222\\ -1.3444\end{bmatrix}$$

$SC_{ration*sire} = 30.2255$

The theoretical difficulties encountered when obtaining sums of squares by differences of submodels, which attempt to obtain Type III sums of squares (Searle, 1972) and are implemented in the Statistical Analysis System (SAS), are resolved by using the direct method in conjunction with the Lagrange multiplier approach. The sums of squares obtained coincide with those reported by Harvey (1960), with discrepancies only due to rounding errors, and correspond to Yates' weighted squares of means (Harvey, 1960; Searle, 1971; Herr, 1986; Steel *et al*., 1997).

With the inverse submatrix $\boldsymbol{G}$ partitioned as in (4), the inverse elements of the reduced submatrices corresponding to each factor can be identified for computing the sums of squares using the direct procedure. For more complex factorial arrays, it is sufficient to define an identity matrix $\boldsymbol{W}$ with the same dimension as $\boldsymbol{G}$ and set to zero the elements of the columns known to be linearly dependent. By computing the matrix product $\boldsymbol{WGW}$, the non-zero elements of the resulting matrix correspond to the required inverse elements, in this case those associated with the mean and the five degrees of freedom of the model. The advantage of this procedure is that all the inverse elements of the matrix $\boldsymbol{X'X}$ are immediately available for calculating the standard errors of the parameter estimators or any linear function of them, thus avoiding computing errors.

CONCLUSIONS

It is feasible and relatively simple to perform analysis of variance in a two-way classification experiment using Lagrange multipliers. The algorithm can be easily implemented in software, reducing the risk of rounding errors. The square segment of the inverse of the coefficient matrix corresponding to the incomplete rank matrix serves as a generalized inverse of that matrix, representing the case in which the imposed restriction is that the sum of the constants within each effect equals zero. This procedure allows all constants and inverse elements required for calculating linear functions to be obtained directly from the model parameter estimators, avoiding semantic and rounding errors associated with sum-and-difference operations between inverse elements in more complex models. It is recommended that the behavior of this procedure be examined in cases involving empty cells.

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HYDROPONIC CULTIVATION EXPERIMENT MONITORED BY AN INTERNET OF THINGS SYSTEM

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ABSTRACT

The application of advanced agricultural technologies such as hydroponics and the Internet of Things (IoT) has the potential to improve cultivation system efficiency and sustainability. This study evaluated the effect of nutrient solution concentration on hydroponic lettuce cultivation (*Lactuca sativa* L. cv Rodhenas). Steiner nutrient solution was used at three concentrations (100, 75, and 50 %, corresponding to treatments T1, T2, and T3, respectively). The experiment was conducted using a completely randomized design, with three treatments and 14 repetitions per treatment, for a total of 42 experimental units. The goal was to reduce the amount of nutrients used while maintaining crop quality and minimizing environmental contamination associated with nutrient leaching. This study also demonstrates the design and implementation of a real-time visualization of variables using IoT technology. The monitoring system was based on a digital temperature and relative humidity sensor connected to a wireless module that transfers data to the cloud, allowing real-time visualization of variable behavior on mobile devices. Cultivation with nutrient solution at 100 % concentration resulted in superior lettuce growth and the highest chlorophyll concentration. This treatment also outperformed the others by nearly 100 g in biomass and showed a significant reduction in fiber content, decreasing to 0.38 % in T2 and 0.46 % in T3.

Keywords: Hydroponics, lettuce, IoT, nutrient solution, chlorophyll.

INTRODUCTION

The global population currently stands at eight billion and is expected to grow by two billion over the next 30 years, requiring a 70 % increase in global food production. This situation calls for radical improvements in the way food is produced and consumed, creating opportunities in urban areas and reducing greenhouse gas emissions through significant changes in traditional agricultural practices (FAO, 2021).

Conventional agricultural practices, including excessive use of water, fertilizers, and pesticides, as well as a high demand for land, result in continuous soil degradation and the depletion of clean water resources, posing significant negative effects on the environment and consumer health (Kazakis and Tsirliganis, 2023). To meet the UN Sustainable Development Goals, the agricultural industry must adapt to modern times by implementing innovative approaches supported by innovative technological and scientific advances, as well as environmentally friendly solutions (Khanal *et al*., 2019).

Agriculture today faces multiple challenges, including land degradation, inefficient management of arable land and water (Mwanake *et al*., 2023), depletion of natural resources, environmental change, meteorological irregularities, pest and disease outbreaks, and increasing food demand and waste (Sharma *et al*., 2022). Addressing these issues requires the adoption of smart agricultural practices and efficient water management techniques (Gupta *et al*., 2020).

Barriers such as limited data processing and storage capacity, along with resistance to technological change, must be overcome to ensure widespread implementation (Symeonaki *et al*., 2022). Adopting new technologies in farming is important to increase productivity, boost economic growth, and make farms more competitive in global markets (Maffezzoli *et al*., 2022; Eze *et al*., 2025).

Protected agriculture involves using controlled environments to maximize crop yields and ensure fruit and vegetable production in the face of current climate change challenges (La Cecilia *et al*., 2023). The Internet of Things (IoT) is an innovative technology that uses intelligent networks, such as the Internet, to virtually connect and communicate with physical objects (Nižetić *et al*., 2020). The interaction of IoT technologies with agriculture could help to ensure a reliable food supply and improve the efficiency of agricultural production processes (Madushanki *et al*., 2019).

Agriculture 4.0, also known as Smart or Digital Agriculture, shares a structural similarity with the concept of Industry 4.0, as it involves the internal and external interconnection of all agricultural production processes. This evolution in agriculture, combined with IoT and data management tools, seeks to address the global challenge of developing sustainable agriculture that meets the food needs of a growing population (Arvanitis and Symeonaki, 2020; Symeonaki *et al*., 2022). Sustainable agriculture and environmental protection can be improved through efficient use of nutrients, accomplished by measuring nutrient availability in the environment and plants (Paul *et al*., 2022).

Hydroponic systems can increase crop growth rates by 10 to 50 % when compared to traditional agricultural practices (Goddek *et al*., 2019), leading to greater productivity and reducing the need for fertilizers. In hydroponic systems, plants develop in controlled, soil-free environments, enabling year-round cultivation (Zhu *et al*., 2023). Hydroponics also has the potential to reduce land, water, and nutrient usage, offering a promising alternative to address global food shortages (FAO, 2021).

Yang *et al*. (2024) installed two hydroponic systems in a controlled-climate greenhouse where they identified water and nutrient absorption patterns for the quality of lettuce grown in these systems during different seasons of the year. They found Deep Water Cultivation (DWC) has a higher yield for total carotenoids, vitamin C, and non-acidified phenols in autumn.

Martínez-Moreno *et al*. (2024) reduced the concentration of Steiner nutrient solution (Steiner, 1961) at the end of the vegetative cycle to decrease nitrate levels in hydroponic lettuce. Reducing the solution concentration from 100 to 50 % decreased nitrate concentration by 14 % in one week and by 22 % in two weeks without affecting vegetative growth.

Furthermore, do Carmo *et al*. (2024) evaluated the growth performance of *Acmella oleracea* L. cultivated in hydroponic systems using nutrient solutions with different electrical conductivity (EC) levels. The results suggest that nutritional management through variations in EC can influence both the growth parameters and the plant's essential oil profile. It was observed that most cultivated species are adversely affected when the conductivity exceeds 2500 $\mu S\ cm^{-1}$.

It is important to consider nutritional performance in cultivated plants, even when attempting to reduce nutrient application costs or environmental impact. Yang *et al*. (2021) evaluated the effect of nutrient solutions with different EC levels while growing *Eruca sativa* Mill. Results showed that applying 1.2 or 1.8 dSm^{-1} improved photosynthetic properties while increasing leaf area, yield, and dry weight.

Other studies evaluated lettuce quality parameters in hydroponic cultivation techniques, including chlorophyll, dissolved solids in the nutrient solution, protein, and fiber content. This crop produced nutritionally superior products compared to conventional cultivation (Ahmed *et al*., 2021). Acharya *et al*. (2021) evaluated the growth of *Spinacia oleracea* L. and *L. sativa* in hydroponic crops compared to conventional crops, where they found higher soluble sugar contents in the soilless cultivated plants, improving the sweetness and texture of green leafy vegetables.

Shin *et al*. (2024) implemented a hydroponic IoT system to supervise and control key plant growth parameters such as pH, EC, and water levels using an ESP32 microcontroller. Hernández-Morales *et al*. (2022) developed and implemented a low-cost IoT system for monitoring microclimates and detecting disease risk factors. The system transmits data to the Internet via the Sigfox network, which includes a cloud-based platform built on microservices and a web application for data analysis and visualization. Meanwhile, Chen *et al*. (2021) developed an IoT system using an STM32 microprocessor to collect real-time data on light, temperature, and humidity, transmitting it via NarrowBand Internet of Things (NB-IoT) to a cloud platform for monitoring and fault detection.

This study presents an innovative hydroponic greenhouse model for lettuce cultivation that integrates advanced technologies and real-time IoT monitoring to optimize resource use and minimize environmental impact. Different nutrient solution treatments were evaluated to determine whether it is viable to reduce the amount of nutrients applied without compromising the quality of the crop. Additionally, a video camera was included for visual crop supervision. It was hypothesized that the concentration of the Steiner nutrient solution has a significant impact on both phenotypic and biochemical parameters, revealing information about the nutritional composition of hydroponically grown lettuce.

MATERIALS AND METHODS

The study was conducted between February and March 2024 at the National Institute of Technology in Celaya, Guanajuato, Mexico (20° 32′ 15″ N 100° 49′ 00″ W). The region features a cloudy and warm climate throughout the year (INEGI, 2020). The average temperature is 18.3 °C, with an average relative humidity of 52.2 % and annual precipitation of 689 mm, typically spread over 65 rainy days, with 5 % rainfall occurring in winter and a photoperiod of 9.2 h per day. The average wind speed during the cultivation experiment was 10.6 km h^{-1}.

The experimental study was conducted in a greenhouse equipped with hydroponic systems (Figure 1A). The structure measured 400 cm in length, 400 cm in width, and 270 cm in height and featured an arched roof covered with a conventional three-layer co-extruded low-density polyethylene (LDPE) white translucent sheet. The greenhouse was fitted with a fixed perimeter and front anti-aphid mesh vents to ensure proper ventilation and pest protection. Inside, it housed three independent hydroponic

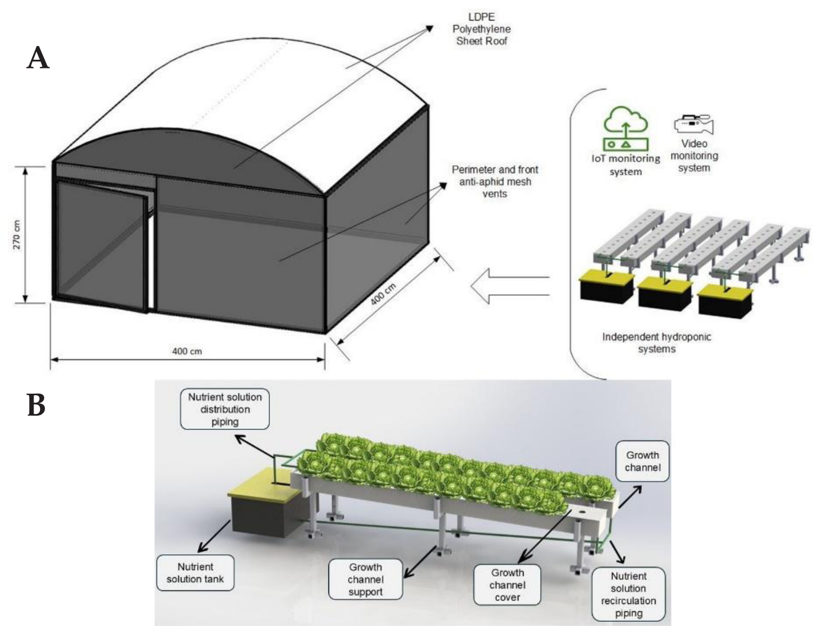

Figure 1. Hydroponic greenhouse system. A: Schematic diagram of the greenhouse and its internal components; B: Hydroponic system model.

systems, an IoT monitoring system that records key environmental variables, and a video monitoring system for continuous visual supervision.

The hydroponic systems were designed and fabricated at the Manufacturing and Prototyping Laboratory of the National Institute of Technology in Celaya. They were constructed using 0.4 cm-thick translucent black polycarbonate sheets (Other), sealed with Pennsylvanian ferrosilicon, and assembled with a heat gun to ensure airtightness and long-lasting durability. Each of the three independent hydroponic systems inside the greenhouse consisted of two polycarbonate channels designed according to plant development parameters, measuring 280 × 25 × 15 cm in length, width, and height, respectively.

The Nutrient Film Technique (NFT) system model (Figure 1B) had a heavy-duty plastic with a capacity of 102 L at the beginning of the channels, equipped with a 25 W submersible pump and a flow rate of 3500 L h^{-1}. The piping was made of Tuboplus hydraulic (PP-R) and included recirculation piping at the end of each channel to facilitate drainage. The channels had three supports that allowed for a 2 % inclination, ensuring a constant and adequate flow of the nutrient solution with a film thickness of 4 to 5 mm. Each of the channels was fitted with covers featuring plant spacings of 18.5 cm.

Cultivation experiment

The hydroponic cultivation experiment for lettuce was conducted in four stages: germination (2 d), root growth (27 d), transplant to the cultivation system and vegetative growth (29 d), and harvest on day 30 after transplanting. The total cultivation duration was 60 d. According to Ruangrak *et al.,* 2023, the nutrient solution was replaced with water for 24 h before cultivation to minimize undesirable flavors in the leaf area associated with the accumulation of salts or mineral compounds, which can alter the sensory characteristics of the final product.

Lettuce seedlings (*Lactuca sativa* L. cv. Rodhenas), measuring 7 cm and exhibiting new leaves and primary root shoots, were positioned in agricultural foam with their roots extending through the bottom and placed into hydroponic baskets. Thirty days after germination (DAG), 30 seedlings per treatment were transplanted into the hydroponic system. This day was considered day zero for monitoring the system. The universal Steiner-type solution (Al Meselmani, 2023) was used, with the constant NFT in the hydroponic system channels. The nutrient formulation was dissolved in 10 L of water with a pH of 6, adjusted using 70 % nitric acid to create a stock solution A-B.

Three nutrient treatments were prepared by diluting the A-B solution at 100 % (T1), 75 % (T2), and 50 % (T3), corresponding to 10, 7.5, and 5 mL per liter of nutrient solution, respectively. The electrical conductivities of these solutions were 1.970, 1.621, and 1.272 dS m^{-1}. T1 served as the control, representing the standard hydroponic formulation that provides a balanced supply of macro- and micronutrients for optimal plant growth (Dasgan *et al*., 2022; Miller *et al*., 2020). The nutrient solution was based on the universal formula proposed by Steiner (1961) with the following concentrations (mg L^{-1}): NO_3, 224; NH_4, 14; P, 47; K, 371; Ca, 180; Mg, 24; S, 66.5; B, 0.25; Fe-EDTA, 2.61; Zn-EDTA, 0.357; Ce-EDTA, 0.179; Mn-EDTA, 1.39; and Mo, 0.11.

The experiment was conducted under a completely randomized design, with three treatments and 14 repetitions per treatment, for a total of 42 experimental units. Prior to analysis, the normality of the data was verified using the Shapiro-Wilk test, and homogeneity of variances was assessed. The data was analyzed using an analysis of variance (ANOVA). When significant differences among treatments were found, Turkey's test ($p \leq 0.05$) was applied. All statistical analyses were performed using Microsoft Excel (version 2021).

Internet of Things monitoring system

During the cultivation experiment, a remote monitoring system based on IoT was used (Figure 2). The system consisted of four components: 1) a digital ambient temperature and relative humidity sensor AM2302 (DHT22, Aosong Electronics Co., Ltd., China); 2) a wireless module based on an ESP8266 NodeMCU development board (Espressif Systems, Shanghai, China); 3) a cloud server using ThingSpeak (The MathWorks Inc., Natick, MA, USA); and 4) a mobile application provided by ThingSpeak for quick data consultations and the MIT App Inventor created by the Massachusetts Institute of Technology (MIT), which features a customized interface for real-time and local data visualization.

The digital sensor AM2302 (DHT22) measured temperature from relative humidity with a sampling frequency of 0.5 Hz and a response time of 2 s. The supply voltage

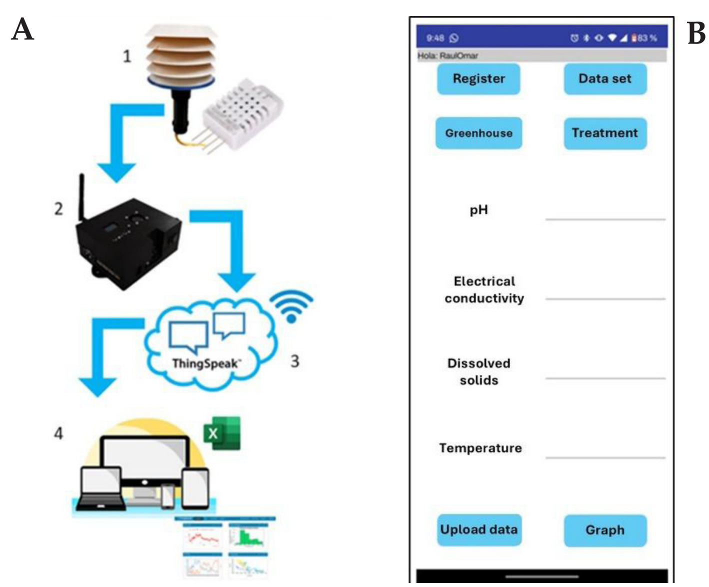

Figure 2. Internet of Things monitoring system. A: Environmental variables monitoring system; B: Mobile application interface used for storing nutrient solution data.

ranged from 3.3 to 5.5 V, and the maximum current consumption was 2.5 mA during measurement and less than 1 mA in idle mode. This sensor acquires ambient temperature and relative humidity data and transmits information to the wireless module every 5 min using the 1-Wire protocol. The sensor's data pin was connected to the general-purpose input/output (GPIO) pin 6 of the ESP8266. The sensor was powered by the development board with 5 V, supplied by the system's main power source.

The sensor data was transmitted to the cloud through a wireless module connected to 2.4 GHz networks. The module featured an I^2C bus general-purpose input/output (GPIO) ports, powered by either 127 V alternating current (AC) or 5 V direct current (DC). It also included a human-machine interface (HMI) to provide users with local real-time information. The data collected by the wireless module was uploaded to the ThingSpeak server via HTTP, where it was stored, analyzed, and visualized. This platform enables remote monitoring via mobile applications, allowing users to access real-time data, view time-series graphs, configure automatic alerts, and receive notifications when conditions exceed set thresholds.

During the experiment, lettuce cultivation in a greenhouse without environmental control was maintained under ideal conditions of 18–26 °C ambient temperature and 55–70 % relative humidity. Each nutrient treatment was monitored daily in the morning for pH, temperature, EC, and dissolved mineral content (ppm) using a C-600 multiparameter water quality meter (Dr. Meter, Shenzhen, China). The manually collected data were stored in a database through the mobile application (Figure 1B) to ensure that the established levels for each treatment were consistently maintained.

The "Invernadero" application was developed specifically for this project using MIT App Inventor (MIT) and is designed to run exclusively on Android devices. The application communicates with the ThingSpeak platform, where the database is organized into three channels (one for each treatment). Each channel contains four fields storing information on pH, temperature, EC, and dissolved mineral content. This setup allows users to visualize and export data, analyze system behavior across treatments, and maintain optimal nutrient solution conditions. The source code for both the system and mobile application is publicly available (https://github.com/DSM40/GreenHouse).

In the hydroponic greenhouse, HD C10_wjmozu-gj WIFI video cameras with integrated microphones were installed at specific points for crop video monitoring. Real-time monitoring is enabled through the Smart mobile application (Tuya Inc., Hangzhou, China), an IoT development platform that allows users to observe the crops remotely from any mobile device. The integrated microphones also provide audio feedback, enabling users to verify the proper operation of the hydroponic systems.

Crop parameters evaluated

Several plant parameters were evaluated, including growth, dry matter, water content, and nutritional profile. The Chlorophyll Content Index (CCI) was evaluated using an SPAD-502 Plus 2900P-C (2900P-C model, Konica Minolta Inc., Osaka, Japan).

Measurements were taken on the leaflets of the third and fourth leaves of the lettuce plants between 9:00 am and 12:00 pm every three days.

Leaf area was determined using an image processing system developed in MATLAB (The MathWorks Inc., Natick, MA, USA) through the App Designer environment. The system integrated the Image Processing Toolbox and the Computer Vision Toolbox, which enabled image segmentation, grayscale conversion, binarization, and pixel counting to calculate leaf area (cm^2) based on a physical reference. Image acquisition was conducted in 10 sessions distributed throughout the growth period. The MATLAB source code for the developed system is available for consultation (https://github.com/DSM40/GreenHouse).

After 30 days in the hydroponic cultivation system, 30 lettuce plants were harvested from each treatment, and the fresh weight of each plant was recorded. Stem diameter was measured at the root insertion point using a Mitutoyo vernier caliper (model 505-742J; Mitutoyo Corporation, Kawasaki, Japan). Root length was determined with a tape measure, and the number of leaves was counted visually.

Lettuce dry weight was measured using 14 replicates per treatment. Leaves and roots were separated, placed in Kraft paper bags, and dried in an FE-131AD oven (Felisa, Mexico) at 72 °C for 48 h. The dry weight was determined gravimetrically using a precision electronic balance with 2000 g capacity and 0.01 g sensitivity (VENOR, analytical laboratory model). Water content was then calculated as the difference between the fresh and dry weights of each sample.

Three plants were randomly selected from each treatment to determine their proximate composition. Samples were taken from the lower, middle, and upper parts of each plant to ensure representativeness. The percentages of moisture, protein, fat, fiber, ash, and carbohydrates present in the crop were determined. Moisture content was obtained by drying the samples in a vacuum oven at 70 °C until a constant weight was achieved, according to AOAC Official Method 934.01 (AOAC, 2000).

Protein content was measured using the Kjeldahl method, AOAC Official Method 984.13 (AOAC, 2000), with an MDK-6 micro-digestion unit (Novatech, Mexico) and applying a conversion factor of 6.25 to transform total nitrogen into crude protein. Ash content was determined by incineration dried samples in an Indet muffle furnace model 273 (Indet, Mexico) at 550 °C for 24 h, according AOAC Official Method 923.03 (AOAC, 2000). Total fat was measured using the Soxhlet extraction method with n-hexane as the solvent, following the AOAC Official Method 920.39 (AOAC, 2000). Carbohydrate content was calculated by difference using the formula described by Fennema (1996).

This experiment followed a completely randomized design, with three replicates per treatment. The experimental unit was the lettuce plant cultivated in the hydroponic system, for a total of nine experimental units. Data normality was verified using the Shapiro-Wilk test, and homogeneity of variances was confirmed. Biochemical variable data were analyzed using analysis of variance (ANOVA), and when significant differences were identified among treatments ($p \leq 0.05$), Tukey's test was applied.

Statistical analysis was carried out using Microsoft Excel (version 2021) with built-in data analysis tools.

RESULTS AND DISCUSSION

Protected agriculture in Mexico covers more than 25 000 ha, with 1728 ha dedicated to hydroponic cultivation (SIAP, 2022). Protected agriculture provides greater environmental and economic benefits than conventional agriculture. However, the country still shows limited development in technologies, tools, materials, and supplies for this sector, leading to a strong dependence on foreign imports. The increasing availability of low-cost electronic components in the national market and on global digital platforms enables the creation of innovative, affordable, and regionally adaptable agricultural technologies. The estimated cost of implementing the proposed system was approximately USD 38, considering low-cost components such as the ESP8266, environmental sensors, and free platforms like ThingSpeak and MIT App Inventor for data development and storage.

Figure 3 illustrates the experimental setup inside the hydroponic greenhouse, including the three nutrient treatments T1 (100 %), T2 (75 %), and T3 (50 %) ,the IoT monitoring system, and the video monitoring camera used for real-time observation of crop development.

Singh and Benbi (2016) reported that the optimal conditions for hydroponic lettuce cultivation include an electrical conductivity (EC) between 1200 and 1800 μS cm^{-1} and a pH of 6–7. Similarly, Samarakoon *et al*. (2019) found that an EC of 1800 μS cm^{-1} in

Figure 3. Experimental setup of the hydroponic greenhouse showing the arrangement of nutrient treatments T1 (100 %), T2 (75 %), and T3 (50 %), the IoT monitoring unit, and the video monitoring system used for data acquisition and environmental control.

nutrient film technique systems enhances photosynthetic activity and fresh weight. However, when EC increased to 2400 μS cm^{-1}, leaf burn occurred without further yield improvement. In contrast, Abou-Hadid *et al*. (1995) observed that lettuce fresh weight decreased when EC exceeded 1000 μS cm^{-1}, although phosphorus and potassium uptake improved at 1800 μS cm^{-1}. These authors recommended maintaining dissolved solid concentrations between 1000 and 1500 ppm to optimize osmotic pressure and promote nutrient absorption through the roots.

In this work, all treatments maintained the recommended conditions from the literature (Figure 4A). In treatment T1, which contained the highest nutrient concentration, EC remained above 100 μS cm^{-1}, which, according to previous reports, can pose a risk to plant development. Moreno-Pérez *et al*. (2015) emphasize the importance of monitoring and recording EC levels, as they can rise to 5000 μS cm^{-1} due to the accumulation of magnesium and calcium salts, negatively impacting nutrient absorption and utilization efficiency.

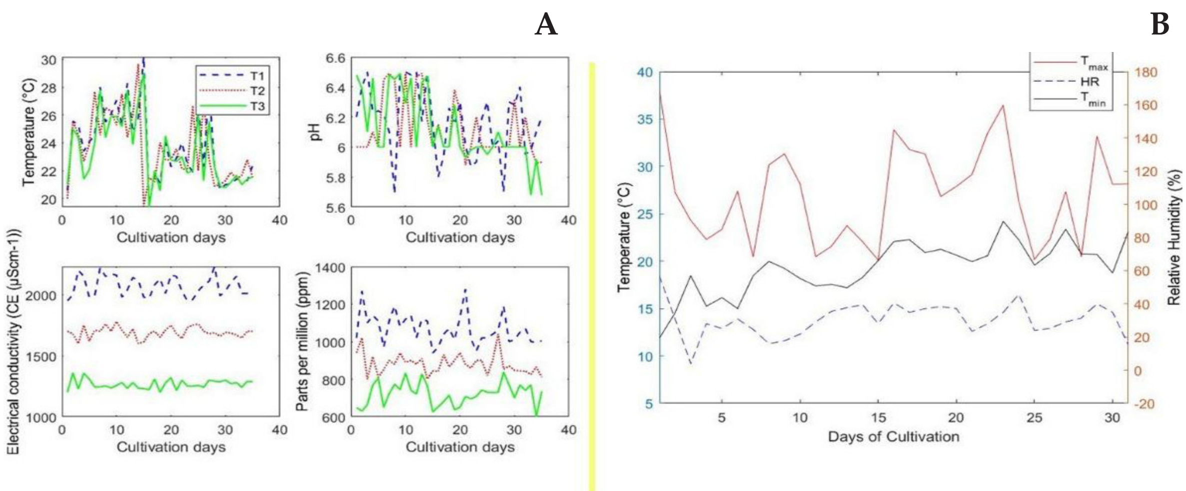

Figure 4. Behavior of the monitored variables in the hydroponic cultivation system. A: Nutrient solution with respect to each treatment; B: Internal environmental variables of temperature (°C) and relative humidity (%) with daily frequency in the Days Post Adaptation (DPA).

Monitoring of physical variables such as relative humidity (%) and temperature (°C) inside the greenhouse was carried out over a 30 d period, producing 6539 consecutive records for each parameter (Figure 4B). Throughout the cultivation experiment, from seedling transplantation to harvest, including the vegetative growth phase, environmental data influencing crop development under actual conditions were collected continuously. The IoT monitoring system enabled real-time tracking and constant access to this information, ensuring precise control of the greenhouse environment.

The recorded data revealed the behavior of ambient temperature and relative humidity, providing a detailed view of thermal fluctuations and their inverse relationship. The average temperature was 21.56 °C, ranging from 13.9 to 29.6 °C, while relative humidity averaged 49.28 %, with values between 11.1 and 87.6 %. Integrating video cameras with the IoT system allowed the surveillance and monitoring of the hydroponic greenhouse, improving security, operational efficiency, and environmental control, guaranteeing greater productivity and sustainability of the hydroponic system.

Crop parameter evaluation results

No significant differences in lettuce morphology were observed among the treatments. All plants exhibited a fresh, vibrant appearance, a characteristic that is highly desirable and valued in this crop. Lettuce is among the most widely consumed vegetables and is generally perceived by consumers as fresh and healthy. This perception is largely influenced by its phenotypic characteristics. In recent years, various technological tools have been developed to monitor lettuce growth and development in greenhouse cultivation (Du *et al*., 2022).

The hydroponic system significantly improved vegetative growth. Leaf area measurements averaged 759.93 mm² for T1, 710.43 mm² for T2, and 715.29 mm² for T3. However, Tukey's test indicated no significant differences in leaf area among the treatments (Figure 5A). T1 exhibited the greatest lettuce fresh weight, with a difference of nearly 100 g compared to the other treatments (Table 1). This difference was statistically significant.

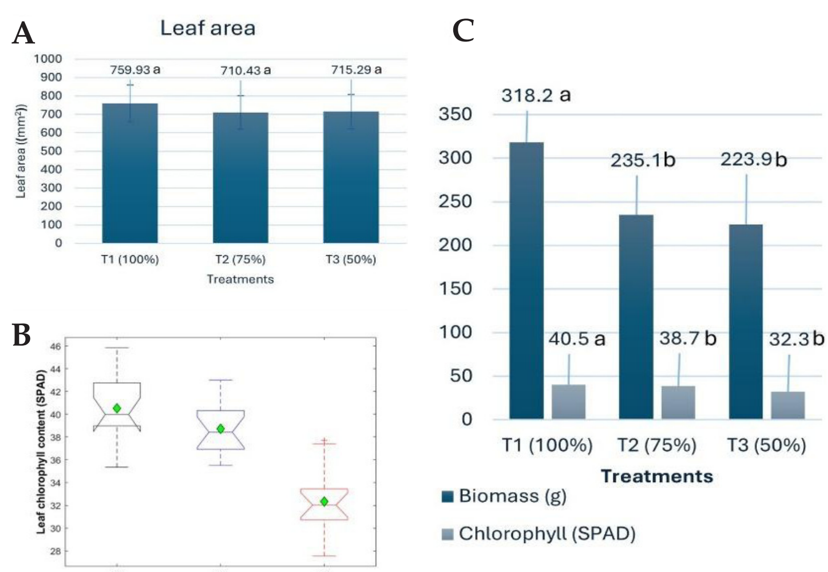

Figure 5. Statistical analysis of phenotypic parameters of lettuce (*Lactuca sativa* L. cv Rodhenas). A: Leaf area of the crop for each treatment; B: Leaf chlorophyll content index for T1 (100 %), T2 (75 %), and T3 (50 %) (mean ± standard error); C: Comparison of arithmetic means of biomass and chlorophyll content in the leaves.

Table 1. Mean fresh and dry weights of aerial and root parts of lettuce plants (*Lactuca sativa* L. cv Rodhenas) under different hydroponic nutrient treatments.

Treatment	Growth parameters			Fresh matter		Dry matter		Number of leaves	Water content (%)
	Leaf height (cm)	Root length (cm)	Stem width (mm)	Leaf weight (g)	Root weight (g)	Leaf weight (g)	Root weight (g)		
T1 (100 %)	23.40 a	16.37 a	22.21 a	329.06 b	24.25 a	10.81 a	0.81 a	27.50 b	95.62 a
T2 (75 %)	22.37 a	14.18 b	21.77 a	244.25 a	24 a	9.06 ab	0.81 a	25.18 a	95.09 b
T3 (50 %)	22.12 a	16.18 a	20.86 a	232.37 a	23.62 a	8.43 b	0.81 a	24.81 a	95.28 ab

Different letters represent significant differences between groups ($p \leq 0.05$).

While chlorophyll content tends to increase with higher EC, elevated EC levels can also cause excessive nitrate accumulation, posing potential health risks if concentrations exceed the permissible limits for fresh produce. Lettuce grown with very high EC values (10 000 μS cm^{-1}) had reduced fresh weight despite an increase in chlorophyll content, which reflects a physiological response to salt stress (Kappel *et al*., 2021). High nitrate levels in vegetables are associated with nitroso compounds, which result in the formation of nitrosamines and methemoglobinemia when metabolized in the human body. Regulating the EC in the nutrient solution is essential to ensure product safety and nutritional quality (Guffanti *et al.,* 2022).

Under the studied conditions, no adverse effects on lettuce growth were observed. The highest nutrient concentration (T1) produced plants with greater fresh weight and chlorophyll content, without visible stress symptoms. Significant differences were detected in growth variables (Table 1), supporting the beneficial effect of optimized nutrient levels. Excessive fertilizer input or salt accumulation in closed hydroponic systems can eventually cause leaf discoloration and tip burn (Samarakoon *et al*., 2019), emphasizing the importance of regular EC monitoring and adjustment.

Treatments T2 and T3 had no significant differences in fresh foliar weight or leaf number, nor did treatments T1 and T3 in root length. However, significant differences were observed between T1 and T3 for dry matter of the leaves (Table 1). This could indicate a deficiency in nutrient absorption due to reduced nutrient concentrations, potentially affecting crop quality and yield. In terms of water percentage, a difference was observed in T2 compared to T1 and T3. However, these variations did not affect crop quality or nutrient absorption. This suggests that the treatment system maintained its efficiency in nutrient delivery. Additionally, the results indicate that T1 promoted more efficient growth, confirming the effectiveness of cultivation under hydroponic conditions.

Lettuce is primarily composed of water, which contributes to its low caloric content. However, its nutritional contribution is significant due to the presence of vitamins, minerals, and bioactive compounds (Madhusudan and Baskaran, 2023). The biochemical parameters obtained from the proximate analyses contributed to determining its composition and nutritional value (Table 2). Although its overall

Table 2. Average biochemical composition (%) of lettuce plants (*Lactuca sativa* L. cv Rodhenas) under different nutrient treatment concentrations.

	Nutritional composition (%)					
Treatment	Moisture	Ashes	Protein	Oils	Fiber	Carbohydrates
T1 (100 %)	95.62 a	0.97 a	0.80 a	0.20 a	1.19 a	1.21 a
T2 (75 %)	95.09 b	0.97 a	0.97 a	0.21 a	1.57 b	1.16 a
T3 (50 %)	95.28 ab	0.89 a	0.90 a	0.13 a	1.65 b	1.13 a

Different letters represent significant differences between groups ($p \leq 0.05$).

nutritional value is relatively low, lettuce contains a high water content (90–95 %) and is a good source of antioxidants, contributing to its health-promoting properties.

The ash, protein, fat, and carbohydrate percentages showed no significant differences between treatments. T1 showed a significant difference for fiber, reducing its percentage to 0.38 % for T2 and 0.46 % for T3. Significant differences were found among treatments in terms of moisture percentage; T1 had the highest, while T2 had the lowest. In contrast, T3 had an intermediate value that did not interfere significantly with either T1 or T2. The weight of the harvested pieces from T2 and T3 (foliar tissue) was not significantly affected by the lower nutrient concentration in the nutrient solution.

During cultivation, chlorophyll content readings were taken every third day until harvest using the non-destructive, dimensionless Soil-Plant Analysis Development (SPAD) method. A significant effect was observed for the interactions between the foliar portion factor (Figure 5B). The readings demonstrate stable values with a low coefficient of variation. The SPAD values were highest in T1 (40.5 ± 1.01), followed by T2 (38.7 ± 0.97) and T3 (32.3 ± 0.97), demonstrating that nutrient concentration significantly affected the chlorophyll levels during the cultivation experiment.

These values align with ranges reported for leafy crops grown under hydroponic conditions. Ahmad *et al*. (2022) demonstrated that SPAD values above 38 are associated with active photosynthetic activity. According to Zhang *et al*. (2022), these chlorophyll values are positively correlated with biomass and yield in lettuce crops. The low value observed in T3 may indicate possible nitrogen or nutrient deficiency, which limited photosynthetic capacity, as noted by Luna-Fletes *et al*. (2023) in their research. Choi *et al*. (2024) validated the use of the SPAD meter as a non-invasive and reliable tool for monitoring crop status. The findings highlight the critical role of maintaining optimal nutrient solution conditions, as the efficient physiological responses in treatments T1 and T2 helped sustain plant balance and vigor.

Cultivation efficiency, assessed through biomass, yielded values of 318.25 g for T1, 235.18 g for T2, and 223.93 g for T3, with no significant difference between T2 and T3 (Figure 5C). The purpose of the greenhouse is to create controlled environmental conditions within a confined space that promote growth and productivity. It is important to monitor, record, and have quick and convenient access to the greenhouse status, which was achieved in this project. The IoT-based environment monitoring system worked efficiently, registering real-time data and providing precise information on environmental variables, ensuring the optimal conditions for hydroponic cultivation development.

CONCLUSIONS

Environmental conditions were continuously monitored using IoT-based devices. The plants exhibited accelerated and vigorous growth, with roots exposed to the nutrient film showing robust and healthy development. No significant differences were found in the nutritional composition of the harvested lettuce among treatments. The IoT monitoring and data recording system functioned reliably throughout the 30-day cultivation period, with no reported failures and constant electronic access to cultivation information. Looking forward, developing a system with failure alarms and automation protocols for correction would be pursued. Overall, these technologies represent an important step toward the development of low-cost, locally adaptable systems that strengthen agricultural innovation and food production efficiency.

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YIELD COMPARISON AND EFFICIENT USE OF NITROGEN IN TWO AQUAPONIC SYSTEMS

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ABSTRACT

The fundamental principle of aquaponics is based on the nitrogen cycle, where fish waste ammonium (NH_4^+) is transformed into nitrites and nitrates, which are absorbed by plants. However, when the pH is greater than 7, NH_4^+ becomes ammonia (NH_3), which can be harmful to fish. In this regard, it was proposed to evaluate the effectiveness of two aquaponic systems consisting of a floating raft and a tezontle bedding in transforming ammonium into nitrates. Two production cycles were conducted. During the first cycle, significant differences among treatments were observed in the leaf area index (LAI), yield, and fresh weight of the lettuce (*Lactuca sativa* L.) plants. In the second cycle, differences were also found in dry weight, width, and length of the leaf (Tukey, α = 0.05). The lettuce yields obtained across treatments during the first production cycle were 3.7 and 3.96 kg m^{-2}, while in the second cycle they increased to 4.05 and 6.8 kg m^{-2}. Nitrogen use efficiency was 49.05 and 38.13 % in the first cycle and 57.86 and 36.6 % in the second cycle. Tilapia (*Oreochromis niloticus* L.) yields were 7.36 and 7.413 kg m^{-3}. The results indicate that the tezontle bedding system was better in terms of yield and other performance indicators.

Key words: *Lactuca sativa* L., *Oreochromis niloticus* L., nitrates, ammonium, nitrites, leaf area index.

INTRODUCTION

Agriculture faces challenges such as climate change, population growth, and environmental pollution. As one of the leading contributors to environmental degradation, this sector has driven the development of sustainable and eco-friendly production systems. Among these, aquaponics has emerged as a promising approach that enables the efficient use of water, soil, and energy resources (Rodríguez-González *et al*., 2017). Aquaponics is a type of circular agriculture that involves closed recirculation of water and nutrients (Lennard and Goddek, 2019). This technique is

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particularly promising in cities, as it can be implemented on a small scale on rooftops, in backyards, and in other small spaces. It also generates sources of proteins, vitamins, and minerals, making it suitable for self-consumption.

Aquaponics integrates aquaculture, which involves the farming of aquatic animals, and hydroponics for the production of vegetables by using the effluent generated in the aquaculture system. The essential components of an aquaponics system are the fish tank, biofilter, and hydroponics unit. The nutrients excreted by the fish or transformed by microbial activity in the fish pond are recirculated throughout the system and assimilated by the plants, while the remaining solid excretions need to be mineralized by microorganisms (Maucieri *et al*., 2018; Li *et al*., 2021).

Nitrifying bacteria that adhere to the biofilter convert waste into nutrients. The more surface area the particles have for bacteria to develop, the more efficient the nitrification process will be at converting ammonium into nitrites and then nitrates. Bacteria break down nitrogen compounds such as ammonium (NH_4^+), ammonia (NH_3), and nitrites (NO_2^-). Through the process of nitrification, these compounds are converted into nitrates (NO_3^-), which serve as essential nutrients for plant growth. This bioconversion ensures that harmful nitrogen species are removed from the effluent, preventing fish poisoning while simultaneously providing plants with the nutrients necessary for development (Li *et al*., 2021; Bautista-Olivas *et al*., 2021).

The hydroponic unit can consist of a floating raft or a substrate bed. Tezontle beddings are commonly used in greenhouse production due to its favorable physicochemical properties: it is a chemically stable material with a near-neutral pH, provides adequate aeration, and exhibits moisture retention capacity that varies with particle size. At the same time, tezontle acts as a biofilter, thus optimizing the space allocated to the system. However, tezontle beddings are difficult to handle and relatively expensive for larger scales. Furthermore, sediment accumulation may lead to clogging, particularly when fish population densities exceed bed load capacity, requiring separate filtration systems. In addition, higher rates of water evaporation and replacement are often required when using tezontle as a substrate (Millares *et al*., 2017).

In contrast, the floating raft method utilizes buoyant sheets made of materials, such as expanded polyethylene or plastic, designed to float on the water surface. Holes are drilled into these sheets to hold the plants, allowing their roots to remain in direct contact with the nutrient-rich water throughout the entire production cycle. This continuous exposure ensures a consistent nutrient supply and promotes uniform plant growth. The floating raft system is widely used in commercial operations and is particularly suitable for leafy crops such as lettuce. Moreover, water loss through evaporation is minimal, reducing the frequency and volume of water replacement required (Li *et al*., 2021).

Tilapia (*Oreochromis* spp.) is the most commonly used fish species in aquaponic systems due to its relatively short production cycle (six to nine months from hatch to harvest), high tolerance to fluctuations in water quality parameters, and resilience to low dissolved oxygen levels (Rodríguez-González *et al*., 2017). On the other hand, lettuce (*Lactuca sativa* L.) is one of the most important horticultural crops due to its short growth cycle (around 30 to 40 d under greenhouse conditions) when cultivated at temperatures between 14 and 24 °C and pH between 5 and 6.8 (Moreno-Simón and Zafra-Trelles, 2014). Several lettuce production cycles can be obtained per tilapia production cycle.

Monsees *et al*. (2019) compared an aquaponic system to a conventional hydroponic control for lettuce cultivation and found differences in the number of leaves and leaf area, with a 32 % lower yield in hydroponics compared to the aquaponic system. Bautista-Olivas *et al*. (2021) reported that lettuce grown in a hydroponic system produced higher yields (1.85 kg m^{-2}) than those grown in an aquaponic system (1.08 kg m^{-2}). This difference was attributed to the immediate availability of nutrients in hydroponics through controlled fertilization. However, hydroponics involves higher fertilizer costs and requires technical knowledge for proper nutrient management, while aquaponics is more environmentally friendly and does not rely on external fertilizer inputs.

The objective of this study was to compare the performance, efficiency of nitrogen utilization, and effectiveness of ammonium (NH_4^+) conversion to nitrates (NO_3^-) in two aquaponic systems used for lettuce and tilapia production. Both systems operated under closed recirculation conditions, one employing a floating raft and the other a tezontle substrate bed. The working hypothesis is that tezontle substrate beds in small-scale aquaponic systems increase nutrient availability, promote ammonium conversion to nitrates, and improve plant growth when compared to floating raft systems.

MATERIALS AND METHODS

The experiment was conducted in a 20 m long and 9 m wide zenithal greenhouse at the Autonomous University of Chapingo (19° 29′ 23′′ N, 98.8° 53′ 37′′ W), at an altitude of 2262 m. A randomized experimental design with three replicates was used, incorporating six experimental units and two treatments.

Treatment 1 (T1): Floating raft. This system consists of three main components: a fish tank with a capacity of 150 L, a plant bed with a volume of 75 L measuring 1.2 × 0.6 × 0.4 m, and a biofilter containing red tezontle with dimensions of 0.79 × 0.5 × 0.27 m and a water retention capacity of 55 L to support the nitrification process (Figure 1A).

Treatment 2 (T2): Tezontle bedding. This system is a simple recirculating aquaponic setup consisting of a 150-L fish tank and a plant bed measuring 1.2 × 0.6 × 0.4 m filled with red tezontle, which has a water retention capacity of 75 L (Figure 1B).

For effluent circulation, each system was equipped with a ¾-inch PVC pipe and a 0.25 HP submersible pump providing an average flow rate of 2.1 L min^{-1}. A GF-370 air pump with a water pressure of 1600 mm was used to maintain continuous aeration for 24 h in both the floating raft plant bed and the fish tank of each system. The experiment began on October 24, 2022, with 16 tilapia (*Oreochromis niloticus* L.) fish per pond,

Figure 1. Aquaponic systems used in the study. A: Treatment 1 (T1), floating raft system; B: treatment 2 (T2), tezontle bedding system.

weighing 158.74 g and measuring 19 cm on average. They were left for a week without lettuce cultivation to allow the systems to stabilize.

On October 31, 21 ‘Ruby Sky’ lettuce seedlings were transplanted into the three replicates of each treatment, for a total of 63 plants per treatment. Harvesting was carried out 28 d after transplanting in both production cycles. The first cycle was harvested on November 28, 2022. The second cycle was transplanted on December 5, 2022, and harvested on January 2, 2023. The fish were harvested 68 d after planting in both treatments.

Throughout the 68-d tilapia production cycle and both lettuce cultivation cycles, weekly monitoring and maintenance activities were conducted to ensure optimal system performance. These activities included fish biometric measurements, plant and water sampling, and daily feeding of the fish with a commercial diet formulated according to recommended nutritional standards (Table 1).

Table 1. Proximate composition and mineral content of the commercial fish feed used during the tilapia (*Oreochromis niloticus* L.) fattening stage.

Feed fattening stage	Protein (%)	Fat (%)	Cu	Fe	Zn (mg kg^{-1})	Mn	B	P (%)	N (%)	S (%)
Nutripec® 35/07	35	7	25.0	464.5	283.25	61.0	123.5	1.59	0.36	0.63
Nutripec® 32/06	32	6	27.5	298.5	281.60	57.5	99.5	1.29	0.25	0.36

Cu: copper; Fe: iron; Zn: zinc; Mn: manganese; B: boron; P: phosphorus; N: nitrogen; S: sulfur. Samples were evaluated at the National Laboratory for Agricultural and Forestry Research and Services (LANISAF) at the Autonomous University of Chapingo, Mexico.

Fish biometry

For 10 weeks, 10 fish per pond were randomly selected, placed in a container with water, and their weight and length were recorded. They were then returned to the fish pond.

Fish feeding

The feed amount was determined weekly based on the total fish biomass (Moreno-Simón and Zafra-Trelles, 2014), considering a feeding rate corresponding to 2 % of the tilapia body weight, following FAO (2023) recommendations. The fish were fed twice daily, divided into two equal rations:

$$AF = B \times TA \times d$$

where *AF* represents the amount of feed, B is the biomass (g), *TA* is the feed rate (%), and *d* is the number of days.

Feed conversion ratio

The feed conversion ratio (FCR) is an indicator of production costs based on the amount of feed consumed by fish in relation to live weight gain throughout the production cycle. A high FCR value indicates high production costs and is therefore unprofitable. According to Salazar-Murillo *et al*. (2023), the feed conversion ratio is calculated as:

$$FCR = \frac{T_{feed}}{\Delta\ Biomass}$$

$$\Delta\ Biomass = B_f - B_i$$

where T_{feed} represents the total feed supplied (kg), B_f is the final biomass (kg), and B_i is the initial biomass (kg).

Growth rate and specific growth rate percentage

According to Moreno-Simón and Zafra-Trelles (2014), the growth rate (GR, cm d^{-1}) and specific growth rate (% GR) are calculated as follows:

$$GR = \frac{L_f - L_i}{T_f - T_i}$$

$$\%\ GR = \frac{ln(L_f) - ln\ (L_i)}{T_f - T_i}$$

where L_i is the initial length (cm), L_f is the final length (cm), T_i is the initial time (d), and T_f is the final time (d).

Plant sampling

Three plants were randomly selected from each experimental unit, for a total of 18 plants sampled per week, for five weeks. The roots were separated from the base of the stem and leaves. Each sample was measured for leaf length and width, root length, and fresh weight of roots and leaves. A LI-COR LI-3100 leaf area integrator was used to obtain the leaf area. The plant material from each repetition was dried in a Binder 108 ED400 oven at 65 °C for 48 h until a constant dry weight was obtained.

Water sampling

To obtain the ammonium (NH_4^+), nitrite (NO_2^-), and nitrate (NO_3^-) content in the effluent and plant bedding, a water sample (50 mL) was taken from the middle of the fish pond and the outlet of the plant bedding. Every week during the lettuce production cycles, a sample was sent to the National Laboratory for Agricultural and Forestry Research and Services (LANISAF) at the Autonomous University of Chapingo.

Nitrogen use efficiency

Nitrogen use efficiency (NUE) was calculated according to the equation proposed by Yang and Kim (2019):

$$NUE = \left(N_f - \frac{N_u}{N_a}\right) * 100$$

where N_f represents the total nitrogen accumulated in plant tissue at the final harvest (g), N_u is the total nitrogen content in the initial plant tissue (g), and N_a denotes the total amount of nitrogen applied (g).

Statistical analysis

Data were analyzed using mean values obtained from each replicate for all evaluated variables. A one-way analysis of variance (ANOVA) was conducted, and mean differences among treatments were determined using Tukey's *post hoc* test at a significance level of $\alpha = 0.05$. All statistical analyses were performed using Minitab version 19.

RESULTS AND DISCUSSION

Lettuce growth performance

Significant differences ($p \leq 0.05$) were found in most growth parameters between treatments (Table 2), indicating that the tezontle bedding system promoted higher photosynthetic activity and greater biomass accumulation. As a result, increased plant size corresponded with higher dry matter production (Escobar-Bahamondes *et*

Table 2. Comparison of means for different growth parameters of lettuce (*Lactuca sativa* L.) at harvest under two aquaponic systems: T1 (floating raft) and T2 (tezontle bedding) across two production cycles.

System	NL	LL (cm)	LW (cm)	LA (cm^2)	LAI	FLW (g)	DLW (g)	Yield ($g\ m^{-2}$)
First lettuce production cycle (October 31, 2022–November 28, 2022)								
T1	17.0[a]	18.40[a]	14.20[a]	2474.35[a]	7.22[a]	135.76[a]	13.93[a]	3959.54[a]
T2	16.0[a]	17.06[a]	13.73[a]	2276.41[b]	6.64[b]	126.96[b]	13.24[a]	3702.87[b]
CV (%)	7.44	5.32	2.39	5.89	5.89	4.74	3.61	4.74
Second lettuce production cycle (December 5, 2022–January 2, 2023)								
T	18[a]	20.30[a]	16.27[a]	3596.15[a]	10.49[a]	208.46[a]	18.95[a]	6079.95[a]
T2	17[a]	17.02[b]	13.98[b]	2578.58[b]	7.52[b]	138.84[b]	14.24[b]	4049.63[b]
CV (%)	5.72	0.12	0.55	12.08	12.08	15.86	12.84	15.86

Means with the same letter in the same column do not represent significant differences according to the Tukey test ($p \leq 0.05$). CV: coefficient of variation. NL: number of leaves; LL: leaf length; LW: leaf width; LA: leaf area; LAI: leaf area index; FLW: fresh leaf weight; DLW: dry leaf weight.

al., 2020). Overall, this system outperformed the floating raft across both production cycles for the variables evaluated.

From the second sampling of the first production cycle, T2 (tezontle bedding) showed a greater number and width of leaves compared to T1 (floating raft). Leaf length in T2 increased significantly 14 d after transplanting and continued to grow until harvest. In the second production cycle, an improvement was observed in all three variables, with T2 consistently outperforming T1.

According to Ahmed *et al*. (2021), increases in leaf area index are influenced by factors such as radiation interception, temperature, water availability, and nutrient supply. In this study, the photosynthetically active radiation (PAR) during the day ranged from 0.14 to 842.45 μmol m^{-2} s^{-1} with an average of 138.78 μmol m^{-2} s^{-1}, and the ambient temperature ranged from 5.8 to 31.9 °C with an average of 17.2 °C. The difference between systems was determined by the effluent composition.

Nitrogen is one of the main nutrients affecting plant development (Bautista-Olivas *et al*., 2021), which can be found in three basic forms: ammoniacal nitrogen, nitrates, and nitrites. However, high levels of ammonium (NH_4^+) and nitrites (NO_2^-) can be toxic to fish and plants (Guzmán-Duchen and Montero-Torres, 2021). However, these compounds remained below 1 mg L^{-1} in both treatments for both production cycles. On the other hand, in aquaponic systems, nitrogen assimilation by plants occurs in the form of nitrates (NO_3^-) and, to a lesser extent, ammonium (NH_4^+) (Benimeli *et al*., 2019). As NH_4^+ is toxic to plant cells, it is converted into organic compounds.

Plants can assimilate high levels of NO_3^-, which are stored in the metabolic reservoir in the cytoplasm and the storage reservoir in the vacuoles. Some species accumulate more nitrates due to highly efficient absorption, limited reduction capacity, or both (Sallenave, 2016). Nitrogen is highly mobile within the plant, and when supply is insufficient, it is redirected to young leaves, causing chlorosis and reduced yield (Lara-Izaguirre *et al*., 2019). For fish, high nitrogen waste levels promote pathogens that reduce growth and overall yield (Ornelas-Luna *et al*., 2017).

The main indicator of aquaponic system performance is the conversion of toxic compounds into plant-available forms, such as NH_4^+ to NO_3^-. In the first lettuce production cycle (weeks 1–5), the floating raft system (T1) had an average NH_4^+ concentration of 0.11 mg L^{-1} in the fish pond. Between weeks five and six, after harvest and without plants, NH_4^+ levels increased in both the pond and growing bed, indicating reduced transformation and lower NO_3^- consumption. This occurs because the absence of plants limits the surface area for nitrifying bacteria and removes the nitrate consumption gradient that drives nitrification, slowing the nitrogen cycle and reducing biofilter efficiency (Bautista-Olivas *et al*., 2021). Conversely, in weeks four and five, NO_3^- levels dropped from 140 to 47 mg L^{-1}, reflecting high nitrate uptake during the final growth stage (Figures 2 and 3).

The initial NO_3^- concentrations in the effluent during the first production cycle were 110 mg L^{-1} for T1 and 90 mg L^{-1} for T2. By the end of the cycle, these values changed to 47.7 and 220 mg L^{-1}, respectively. A similar trend was observed in the second production cycle, with average NO_3^- concentrations of 135.26 mg L^{-1} for T1 and 173.9 mg L^{-1} for T2 (Figures 2 and 3).

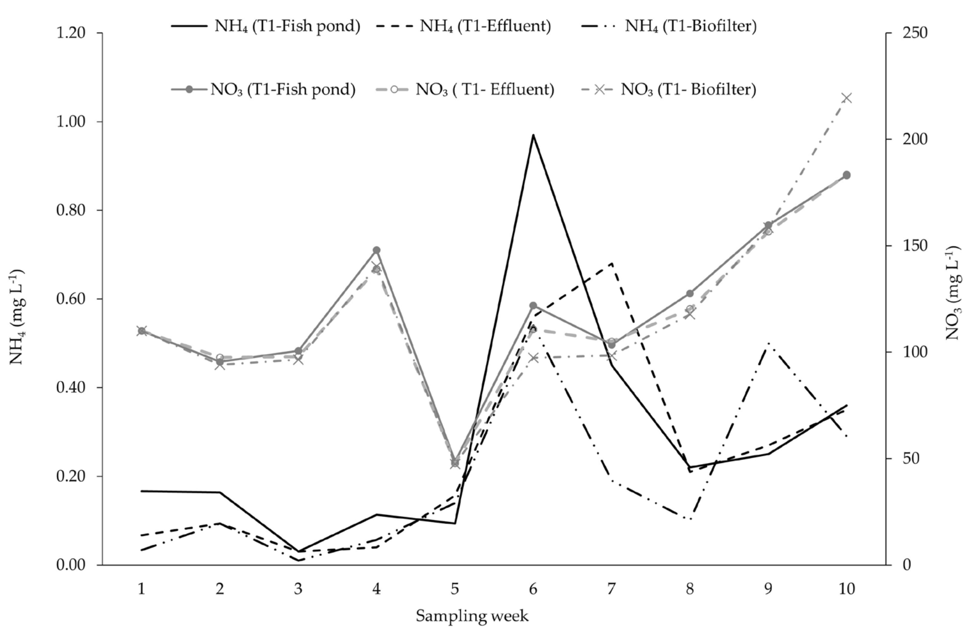

Figure 2. Dynamics of ammonium (NH_4^+) and nitrate (NO_3^-) concentrations in the effluent, fish pond, and biofilter during the tilapia (*Oreochromis niloticus* L.) production cycle in the T1 (floating raft) system.

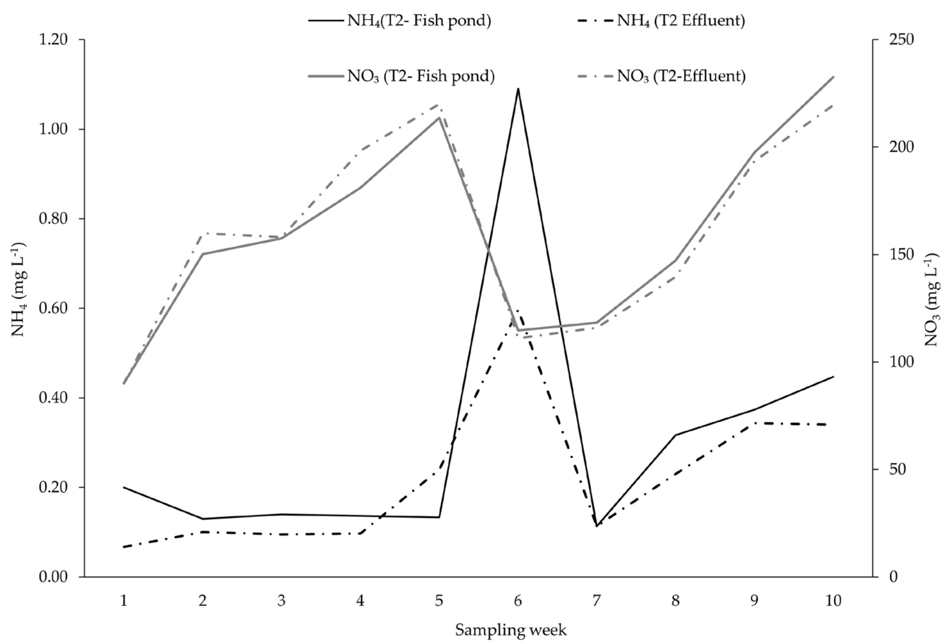

Figure 3. Dynamics of ammonium (NH_4^+) and nitrate (NO_3^-) concentrations in the effluent and biofilter during the tilapia (*Oreochromis niloticus* L.) production cycle in the T2 (tezontle bedding) system.

During the second production cycle, initial NH_4^+ concentrations in the fish pond were 0.17 mg L^{-1} for T1 and 0.2 mg L^{-1} for T2, rising to 0.35 and 0.45 mg L^{-1} by the end of the harvest. The maximum average NH_4^+ levels in the effluent for both cycles and treatments remained within recommended limits (<1 mg L^{-1}) (Somerville *et al*., 2014). The tezontle bedding system supported better microbial development and more efficient nitrification compared to the floating raft.

Tilapia farming

The daily feed conversion rate depends on both the weight and number of fish in the pond (Moreno-Simón and Zafra-Trelles, 2014). This indicator provides a measure of feed efficiency, allowing rations to be adjusted to ensure proper consumption, reduce energy waste, and maximize return on investment. Overfeeding can lead to increased excretion of protein-rich urine and feces, which upon decomposition produce toxic nitrogenous compounds, such as ammonium (FAO, 2023).

An adequate feeding regimen ensures that fish receive the necessary nutrients without overfeeding, which directly affects fish and plant health, water quality, productivity, and overall system profitability. Fish feed represents a major production cost, accounting for approximately 60 % of tilapia production expenses (Zafra-Trelles *et al*., 2019). The daily feed calculation resulted in an initial weekly feed portion of 355.96 g for T1 and 355.21 g for T2, and a final weekly amount of 511.25 g for T1 and 510.23 g for T2. However, the actual average feed consumed during the production cycle was only 62.31 % for T1 and 63.04 % for T2 of the calculated amounts, indicating relatively low feed intake in both treatments.

Production efficiency was evaluated using the feed conversion ratio (FCR). In this study, FCR values of 1.91 and 1.96 were obtained for T1 and T2, respectively. Although these values fall within the range between 1.39 and 2.14 reported by Zafra-Trelles *et al*. (2019), there is room for improvement, as nearly 2 kg of feed are required to produce 1 kg of tilapia, resulting in higher production costs.

The growth rate (GR) and percentage of specific growth rate per day (% GR) observed in this study were relatively low compared to values of 0.27 and 2.4 % d^{-1} reported by Hernández-Naranjo *et al*. (2021). Tilapia in T1 showed a slightly higher GR of 0.2 d^{-1} and % GR of 0.01 % d^{-1} compared to T2, which had a GR of 0.19 d^{-1} and % GR of 0.009 % d^{-1} throughout the production cycle. Overall, both treatments exhibited low specific growth rates.

In the analysis of variance in treatments T1 and T2, no significant differences ($p \leq 0.05$) were found for fish in terms of weight (228.24 and 227.78 g $fish^{-1}$), length (22.91 and 22.83 cm), and yield (7.41 and 7.36 kg m^{-3}). From the initial to the final week, the average weight gain was 69.04 g in T1 and 69.5 g in T2, which is reflected in the yield values. However, fish in T2 showed a slightly better response across the measured variables, likely due to the larger biofilter area in this system, which improved nitrification, nutrient conversion, and overall water quality.

The low tilapia production observed, likely due to reduced feed consumption, can be attributed to factors affecting water quality, including dissolved oxygen (DO), pH, temperature, and concentrations of NH_4^+, NO_2^-, and NO_3^-. Dissolved oxygen remained within optimal levels (6–8 mg L^{-1}), preventing competition for oxygen. According to Aleksić and Šušteršič (2020), a pH range of 6–8.5 is optimal for fish growth and the nitrification process, while nutrient absorption by plants is effective at slightly lower pH values (5.5–7.5). In this study, T1 had an initial pH of 7.85 and a final pH of 6.21, averaging 6.3, whereas T2 ranged from 7.7 to 6.21, with an average of 6.32. Although these values fall within the optimal range for plant nutrient uptake, they were near the lower limit for tilapia growth in both treatments.

During the first production cycle, average water temperatures in the fish pond were 28.17 °C during the day and 22.3 °C at night, while in the second cycle they averaged 27.19 °C (day) and 21.86 °C (night). According to Sallenave (2016), tilapia require temperatures between 27.8 and 29 °C during winter to achieve optimal growth. Rodríguez-González *et al*. (2017) found that temperature fluctuations can cause behavioral changes in fish, leading to slower growth, reduced feed intake, and altered activity of nitrifying bacteria, which in turn affects nutrient availability and the quality of both lettuce and fish. In this study, suboptimal temperatures occurred only at night, when fish are not feeding, and therefore likely did not significantly impact overall production.

During the tilapia production cycle, NO_2^- concentrations in the fish pond for T1 remained at 0.8 mg L^{-1} during the first three weeks, decreased to 0.47 mg L^{-1} in week four, and returned to 0.8 mg L^{-1} by the end of the cycle, resulting in an average concentration of 0.76 mg L^{-1}. In T2, NO_2^- levels ranged from 0.23 to 0.47 mg L^{-1}. The maximum average concentrations of NH_4^+ and NO_2^- in the fish pond for both treatments were within the recommended limits reported by Aleksić and Šušteršič (2020) (Table 3).

Table 3. Range of ammonium (NH_4^+), nitrite (NO_2^-), and nitrate (NO_3^-) concentrations in the effluent and fish pond during two lettuce (*Lactuca sativa* L.) production cycles for T1 (floating raft) and T2 (tezontle bedding), compared with recommended values.

(mg L^{-1})	Cycle 1		Cycle 2		Recommended values*
	Effluent				
	T1	T2	T1	T2	
Ammonium	0.02–0.16[b]	0.07–0.24[a]	0.21–0.68[a]	0.11–0.60[a]	<1
Nitrites	0.47–0.80[a]	0.27–0.47[b]	0.63–0.80[a]	0.23–0.47[b]	<1
Nitrates	47.25–139.05[b]	90–220.07[a]	105.07–183.50[b]	111–219.50[a]	5–150
	Fish pond				
Ammonium	0.03–0.17[b]	0.13–0.20[a]	0.22–0.97[b]	0.11–1.09[a]	<1
Nitrites	0.47–0.80[a]	0.27–0.47[b]	0.63–0.80[a]	0.23–0.47[b]	<1
Nitrates	48.63–147.95[b]	90–213.60[a]	103.53–183[b]	114.67–232.50[a]	5–150

Means with the same letter in the same row do not represent significant differences according to the Tukey test ($p \leq 0.05$). *Aleksić and Šušteršič (2020).

The maximum nitrate concentration in the nutrient solution in T1 during both production cycles remained above 140 mg L^{-1}, while in T2 the maximum nitrate concentration in the effluent remained above 220 mg L^{-1}. Although nitrate concentrations in T1 stayed within the recommended ranges (Table 3), Sallenave (2016) found that effluent nitrate levels above 90 mg L^{-1} do not pose toxicity risks, as plants can store excess nitrate until needed. The NH_4^+ concentrations in the fish pond over the 10-week tilapia production cycle ranged from 0.17 mg L^{-1} (initial) to 0.36 mg L^{-1} (final) in T1, with a spike to 0.97 mg L^{-1} in week six, coinciding with the absence of plants. In T2, NH_4^+ started at 0.2 mg L^{-1} and increased to 0.45 mg L^{-1} by the end of the cycle, showing a similar peak of 1.09 mg L^{-1} in week six.

In terms of nitrogen use efficiency (NUE), the values obtained in this study were similar to those reported by Zou *et al*. (2016), who found efficiencies of 53.3 and 56.6 % in aquaponic systems supplemented with nitrifying bacteria, highlighting enhanced bacterial activity in nitrogen transformation. The results also exceeded those reported by Yang and Kim (2019), who recorded a NUE of 30.7 % when evaluating three feeding strategies (progressive, intermediate, and uniform). In their study, feeding rates were below optimal levels for maintaining suitable water quality and fish growth: the progressive feeding strategy increased feed proportionally to fish weight, the intermediate increased it by 0.7 %, and the uniform treatment maintained a constant feed rate throughout the production cycle.

In this study, maximum NUE values of 57.2 % were obtained for T1 and 46.95 % for T2 during the first production cycle, while values of 57.86 and 36.86 % were recorded for the second cycle, respectively. These results indicate superior performance for the T1 system in both production cycles. According to Zou *et al*. (2016), higher NUE values are typically associated with greater abundance and activity of nitrifying bacteria, which are favored by increased oxygen availability. Similarly, Yang and Kim (2019) reported that uniform feeding enhances nutrient availability, improves water quality, and consequently increases plant quality and yield. In the present study, feed quantities were calculated based on fish biomass; however, actual feed consumption was slightly lower than the estimated amount.

Lettuce yields during the first production cycle were higher in T2 (3.96 kg m^{-2}) compared to T1 (3.7 kg m^{-2}) (Figure 4A). Similarly, in the second production cycle

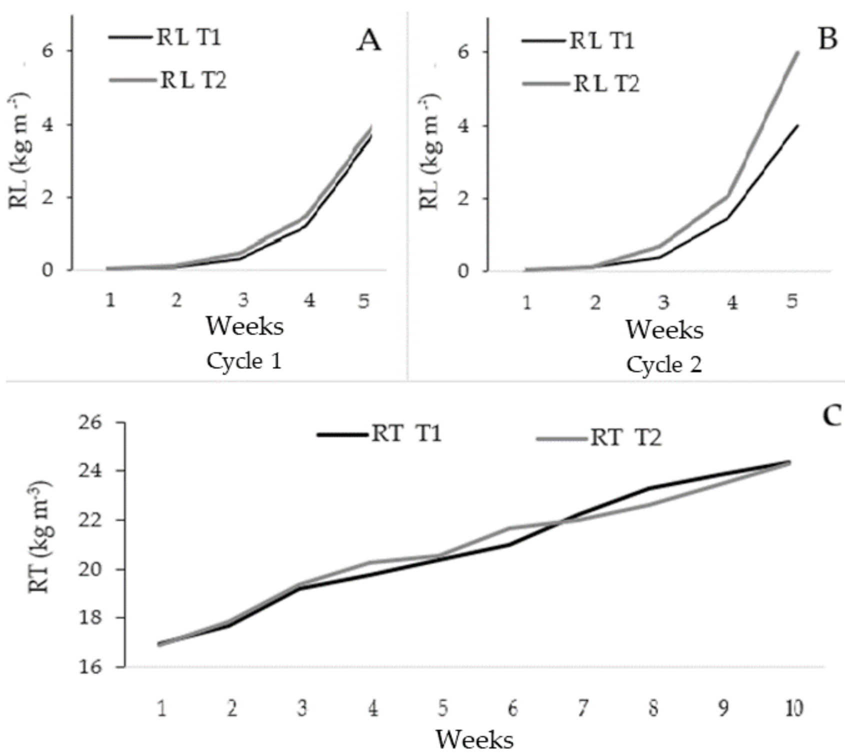

Figure 4. A: Lettuce (*Lactuca sativa* L.) yield (RL) in the first production cycle; B: Lettuce yield (RL) in the second production cycle; C: Tilapia (*Oreochromis niloticus* L.) yield (RT) over the whole production cycle.

(Figure 4B), T2 achieved a higher yield (6.08 kg m^{-2}) than T1 (4.05 kg m^{-2}), indicating a more efficient nitrification process and enhanced nitrate uptake in the T2 system (Table 2). Although plants exhibited satisfactory growth and development in both treatments, the superior performance of T2 suggests greater nitrate absorption and improved nitrogen use efficiency. According to Lara-Izaguirre *et al*. (2019), nitrogen is a key element regulating plant growth and development, primarily assimilated in the form of nitrates.

The lettuce yields obtained in both treatments in this study were higher than those reported by Jordan *et al*. (2018) for a hydroponic system (2.88 kg m^{-2}). Similarly, Bautista-Olivas *et al*. (2021) found a lettuce yield of 1.85 kg m^{-2} for hydroponic and 1.08 kg m^{-2} for aquaponic conditions, managed a biomass of 1.5 kg m^{-3} in fish, and 19 lettuces per m^2. In contrast, Jordan *et al*. (2018) obtained better results in hydroponics, which was due to the use of fertilizers in the nutrient solution and the elevated ambient temperatures during the experimental period (June–July). These conditions likely increased thermal stress on the fish, reducing feed intake and consequently lowering the organic load in the nutrient solution, which limited nutrient availability for plants. Lennard and Goddek (2019) compared lettuce growth under hydroponic and aquaponic systems across different seasons (summer, winter, and spring), using a density of 15 plants per m^2. In all cases, lettuce performance in the aquaponic system was comparable to that in the hydroponic system. However, during winter, the hydroponic setup yielded better results due to reduced fish feeding activity and lower water temperatures, which limited nutrient availability in the aquaponic system. Similarly, Barbosa *et al*. (2020) evaluated an aquaponic system to compare tilapia and lettuce yields under varying fish tank volumes, with an initial fish biomass of 4 kg m^{-3} and 19 lettuce plants per m^2. They found no significant differences in lettuce yield or fish performance among the treatments, which may be due to consistent water quality and uniform fish stocking density across systems. In this work, the increase in tilapia biomass throughout the production cycle rose from 16.93 to 24.29 kg m^{-3} in T1 and 24.34 kg m^{-3} in T2 (Figure 4C).

The weight and biomass of vegetables depend on several factors, including the type of substrate used in aquaponic cultivation, the season, the type of aquatic organism produced in aquaponics, and the quantity and quality of the feed (Barbosa *et al*. 2020). The pH of the water, electrical conductivity, ammonium, dissolved oxygen, and water temperature must also be controlled, as these factors directly affect the nutrient solution and influence the development of fish and plants (Somerville *et al*., 2014). The yields obtained in this study for both tilapia and lettuce were compared with those reported by other authors (Table 4). Among the treatments evaluated, T2 (tezontle bed) demonstrated the best overall performance.

Table 4. Comparative yields of lettuce (*Lactuca sativa* L.) and tilapia (*Oreochromis niloticus* L.) obtained in this study and reported by other authors under different production systems and environmental conditions.

Author	Production system	Location		Yields		
				Lettuce (kg m^{-2})		Tilapia (kg m^{-3})
Lennard and Goddek (2019)	Aquaponics Hydroponics	Australia			5.77 5.46	-
Jordan *et al*. (2018)	Aquaponics Hydroponics	Brazil			2.58 2.88	-
Barbosa *et al*. (2020)	Aquaponics	Brazil			1.62 1.42	4.44 4.88
Bautista-Olivas *et al*. (2021)	Aquaponics Hydroponics	Mexico			1.08 1.85	-
This study (2022–2023)	Aquaponics	Mexico	1st cycle	T1 T2	3.70 3.96	7.36 7.41
			2nd cycle	T1 T2	4.05 6.80	

CONCLUSIONS

The results showed that the tezontle bed is the most effective alternative for small-scale aquaponic systems, as it promotes nutrient availability, the conversion of ammonium to nitrates, and superior vegetable growth compared to the floating raft system, thus partially confirming the proposed hypothesis. In contrast, the floating raft treatment required additional nutrients and a more efficient biofilter to achieve optimal nitrogen transformation and system stabilization, limiting its productive performance.

The separation of the biofilter from the growing bed in the floating raft system reduced nitrate availability and leaf area development. For the aquaculture component, fish growth, weight, and survival did not differ significantly between treatments; however, fluctuations in water pH affected overall performance, showing the importance of continuous monitoring to ensure balanced conditions for both fish and plants.

Although the floating raft treatment had higher nitrogen use efficiency, it did not result in increased biomass accumulation, suggesting possible nitrogen retention in plant tissues and incomplete transformation for growth. Future studies should examine nitrogen assimilation and accumulation dynamics to optimize nutrient utilization and enhance the integrated productivity of aquaponic systems.

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Agrociencia

POSTHARVEST TECHNOLOGIES FOR FRUITS AND VEGETABLES: CURRENT STATUS AND PROSPECTS

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ABSTRACT

Fruits and vegetables are susceptible to water loss and other physiological and pathological disorders, leading to substantial postharvest losses. These problems have led to the creation of new treatments and preservation technologies that can keep products fresh for longer. In recent years, there has been a growing interest in sustainable technologies that are safe for consumers and leave no toxic residues. These systems include chemical methods, such as ozone, electrolyzed water, hydrogen-rich water, essential oils, and vegetable extracts, as well as physical methods like controlled and modified atmosphere storage, irradiation, and the use of edible films and coatings. Additionally, cold plasma treatment is an emerging technology under active investigation for its potential applications in food preservation. The objective of this review is to present and examine emerging and advanced technologies designed to enhance the preservation and extend the shelf life of fruits and vegetables during the postharvest period.

Keywords: food safety, microbial control, physicochemical preservation, sensory quality, shelf-life extension.

INTRODUCTION

Fruits and vegetables, rich in vitamins, antioxidants, minerals, and fiber that contribute to a balanced diet and optimal health, are increasingly in demand due to the growing consumer preference for foods with high nutritional value. More than 675 megagrams (Mg) of fruits are produced worldwide each year (Pereira *et al*., 2022). Approximately one-third of the world's production for human consumption, or around 1.3 billion Mg of food, is lost and wasted annually (Moračanin *et al*., 2023). Food losses occur throughout the stages of agricultural production, postharvest handling, and storage, as well as during processing and packaging. In contrast, food waste primarily takes place during the distribution and consumption phases (Gustavsson *et al*., 2011; Elik *et al*., 2019).

A major obstacle to global food security is the postharvest loss of fruits and vegetables, estimated to account for approximately 50 % of total worldwide production. In developing countries, approximately 25–50 % of fruit and vegetable production is lost, primarily due to inadequate handling, poor storage and transportation infrastructure, and the use of insufficient or inappropriate packaging (Elik *et al*., 2019; Tapsoba *et al*., 2022). In contrast, postharvest losses of fruits and vegetables in Europe are estimated to be around 27 %. In developed countries, most losses occur beyond the farm gate, largely resulting from microbiological and physiological deterioration associated with natural senescence, as well as technological, planning, and logistical shortcomings. However, the most significant losses take place during the distribution and consumption stages (food waste), accounting for approximately 46 % (Bennett *et al*., 2011; Elik *et al*., 2019).

Refrigeration has been used for centuries as one of the simplest and most practical methods for preserving fruits and vegetables. However, temperature control alone is often insufficient, particularly given the long distances that food products are transported today. To address this limitation, additional preservation strategies have been developed, including chemical treatments, ultraviolet irradiation, controlled and modified atmosphere storage, edible films and coatings, and, more recently, cold plasma technology (Sandarani *et al*., 2018; Palumbo *et al*., 2022).

Chemical treatments, such as hydrogen peroxide, ozone, and electrolyzed water, are primarily effective in maintaining the microbial quality of fresh produce. Controlled atmosphere storage and modified atmosphere packaging help reduce metabolic activity, thereby extending the shelf life of fruits and vegetables. Irradiation and cold plasma have also proven effective against a wide range of pathogenic microorganisms. Additionally, edible films and coatings can minimize weight loss and metabolic activity in fruits and vegetables, thus preserving their overall quality (Palumbo *et al*., 2022; Wu *et al*., 2024). This review aims to provide an overview of emerging and advanced postharvest treatments and technologies for fruits and vegetables, highlighting their effectiveness, applications, and potential to extend shelf life.

CHEMICAL AND NATURAL TREATMENTS

Synthetic chemical products

The most widely used industrial control system to prevent microbial spoilage and maintain postharvest shelf life is the application of synthetic chemical products. Fungicides such as imazalil and thiabendazole, as well as sanitization with sodium hypochlorite, are particularly notable. However, legislative restrictions in many countries are increasingly limiting their use due to concerns over the emergence of resistant pathogen strains and environmental and food safety issues (Lado *et al*., 2013; Murray *et al*., 2019). Sanitization is considered effective and safe when the agent achieves a 5-log (99.999 % reduction) in bacterial concentration and a 4-log (99.99 % reduction) in mold concentration (Frisón *et al*., 2013).

Ozone

Ozone is a highly effective disinfectant with extremely rapid antimicrobial action. It is an unstable molecule composed of three oxygen atoms and readily decomposes into oxygen (O_2) and nascent oxygen. Ozone does not produce toxic byproducts and is safe, efficient, and environmentally friendly, leaving only oxygen as a residue, which makes it a suitable alternative to chlorine (Frisón *et al*., 2013). To inactivate microorganisms on fruit, high doses of ozone are required, as the organic components of the fruit react easily with ozone, reducing its sanitizing effectiveness. However, excessive ozone must be avoided, as it can damage the tissues of the product and compromise its quality.

Due to the above, it is advisable to carry out prior studies of the optimum ozone doses for each product (Bataller-Venta *et al*., 2010). In cantaloupe melon (*Cucumis melo* L.), gaseous ozone (0.15 ppm during the day and 0.3 ppm at night) maintained firmness and decreased the concentration of ethylene and mesophilic aerobic bacteria, in addition to reducing the enzymatic activity of α-arabinopyranosidase, β-galactopyranosidase, and polygalacturonase from the third day of treatment (Toti *et al*., 2018). In coriander (*Coriandrum sativum* L.), ozone (0.68 mg L^{-1}) maintained physical appearance and suppressed respiration rate and polyphenol oxidase activity and inhibited the activity of chlorophyll-degrading enzymes (Xu *et al*., 2019).

Electrolyzed water

Electrolyzed water is generated by the electrolysis of an aqueous sodium chloride solution (0.5–1 % NaCl). This process produces two types of electrolyzed water: acid electrolyzed water (AEW), also known as oxidizing electrolyzed water, and neutral electrolyzed water (NEW). AEW is produced at the anode, while NEW is generated at the cathode. NEW has a low pH (2.1–4.5), a high oxidation-reduction potential (above 1000 mV), and the presence of hypochlorous acid, which confers a strong bactericidal effect against pathogenic microorganisms and fungi responsible for postharvest diseases (Rico *et al*., 2007; Selma *et al*., 2008). Meanwhile, the oxidation-reduction potential in AEW ranges from 500 to 700 mV, and the pH ranges from 5 to 8.5, resulting in a strong bactericidal effect (Rico *et al*., 2007; Ramos *et al*., 2013).

Both AEW and NEW have shown similar efficacy to hypochlorite, at equivalent free chlorine concentrations, against *Escherichia coli*, *Listeria innocua*, and *Salmonella choleraesuis* in apple (*Malus domestica* L.) slices artificially inoculated with these bacteria (Graça *et al*., 2011). Other studies have demonstrated the effectiveness of electrolyzed water as a disinfectant against *E. coli* in cut apple and melon (Wang *et al*., 2006), *Botryosphaeria berengeriana* in cut pear (*Pyrus communis* L.) (Al-Haq *et al*., 2002), and *Penicillium expansum* during apple handling and processing (Okull and Laborde, 2004).

Hydrogen-rich water

Hydrogen-rich water (HRW) is a practical and safe alternative to hydrogen gas for various applications, including postharvest treatment of fruits and vegetables (Dong *et al*., 2023a). Many physiological and developmental processes, including the postharvest preservation of horticultural products, depend on gas transfer molecules such as hydrogen sulfide (H_2S) and methane (CH_4) (Fang *et al*., 2021). Several studies have demonstrated the beneficial effects of H_2S (Hu *et al*., 2014) and nitric oxide (NO) (Lai *et al*., 2011) in extending the shelf life of strawberries (*Fragaria* × *ananassa* Duch.) and tomatoes (*Solanum lycopersicum* L.), respectively. Furthermore, H_2 can regulate the antioxidant defense system, mitigating abiotic stresses such as high salinity and low temperature. Additionally, H_2 is involved in regulating postharvest biochemical and physiological behavior.

In kiwifruit (*Actinidia deliciosa* Liang), HRW reduces lipid peroxidation and delays the ripening process, thus maintaining quality and extending postharvest life (Hu *et al*., 2014). A 50 % HRW solution was found to effectively maintain the chlorophyll content in pak choi (*Brassica rapa* subsp. *chinensis* L.), and when combined with vacuum precooling, it also helped maintain antioxidant compounds while reducing the rate of weight loss (An *et al*., 2021). In okra (*Abelmoschus esculentus* L.), HRW maintained cell wall biosynthesis by regulating genes related to cellulose, hemicellulose, and pectin at different times during storage, which helped the fruit to retain its firmness and delay softening (Dong *et al*., 2023a).

One disadvantage of conventional HRW is the short residence time of H_2 in water, as well as its low solubility. However, nanobubbles are an emerging technology that can increase the residence time and content of H_2 in water. Hydrogen nanobubble water (HNW) has been applied in preharvest and other areas, such as water treatment. The vase life of cut carnation flowers (*Dianthus caryophyllus* L.) was prolonged by regulating nuclease and protease activities through the postharvest application of HNW (Li *et al*., 2021). However, further research is needed to evaluate the effectiveness of this technology for postharvest applications in other horticultural products.

Essential oils and plant extracts

Synthetic fungicides are associated with environmental and human health risks. To replace chemical treatments, essential oils (EO) and plant extracts have emerged as safe alternatives over the last two decades. Plants contain bioactive compounds that exhibit biological activity against pathogenic microorganisms, including phytopathogenic fungi, oomycetes, and bacteria. With more than 250,000 plant species worldwide, plant extracts and essential oils offer considerable potential for postharvest applications in a variety of fruits and vegetables (El Khetabi *et al*., 2022). Essential oils are produced as secondary metabolites in various parts of aromatic plants, including leaves, fruits, seeds, flowers, stems, roots, buds, and bark (Aftab and Hakeem, 2021). However, EOs can be photodegradable and volatile, which may reduce their long-term effectiveness. As a result, EOs are often tested in combination with other technologies (Table 1).

Table 1. Recent studies on essential oils (EOs) and plant extracts as postharvest treatments for fruits and vegetables.

Product	EO Treatment	Results	Limitations/notes	References
Postharvest washing of fresh produce	Thyme (*Thymus vulgaris* L.), oregano (*Origanum vulgare* L.), cinnamon (*Cinnamomum verum* J. Presl), and clove (*Syzygium aromaticum* L.)	Reduced foodborne pathogens, strongly inhibiting *E. coli*, *Salmonella*, and *L. monocytogenes*.	Need for standardization of EO concentrations and application methods.	(Pizzo *et al*., 2023)
Apple (*Malus domestica* L.)	Lemongrass (*Cymbopogon citratus* (DC.) Stapf) with poly(lactic acid) nanocapsules	Treated fruits showed smaller bitter rot lesions compared to non-capsulated EO and control.	Potential cost and complexity of nanocapsule production.	(Antonioli *et al*., 2020)
Jackfruit (*Artocarpus heterophyllus* Lam.)	Vetiver (*Vetiveria zizanioides* L.) and basil (*Ocimum basilicum* L.)	Reduced rot caused by *P. notatum* and *R. microsporus* on the jackfruit surface.	Requires further testing for commercial application.	(Atif *et al*., 2020)
Guava (*Psidium guajava* L.)	Gum Arabic (GA) with cinnamon	10 % GA + 1 % cinnamon EO preserved firmness, color, pH, flavor index, SSC, carotenoids, and chlorophylls during cold storage.	Synergistic effects of GA and EO need more exploration.	(Etemadipoor *et al*., 2019)
Strawberry (*Fragaria* × *ananassa* Duch.)	Chitosan/citrus /silver nanoparticle films and gamma irradiation	Composite films showed less weight loss than controls. Gamma irradiation reduced firmness and decay over 12 d.	Combined effects of EO, chitosan, and nanoparticles need further study	(Shankar *et al*., 2021)
Peach (*Prunus persica* L.)	Lavender (*Lavandula angustifolia* Mill.), rosewood (*Aniba rosaeodora* Ducke), dill weed (*Anethum graveolens* L.), and fennel (*Foeniculum vulgare* Mill.)	Rosewood EO in vapor phase effectively reduced decay and maintained weight loss and total soluble solids at room temperature and refrigeration.	Variability in effectiveness depending on EO concentration and application method.	(Lin *et al*., 2022)
Dragon fruit (*Selenicereus undatus* (Haw.) D.R. Hunt)	Ginger (*Zingiber officinale* Roscoe), turmeric (*Curcuma longa* L.), and dukung anak (*Alpinia galanga* L.) extracts	10 g L^{-1} controlled anthracnose; higher concentrations (≥15 g L^{-1}) may cause phytotoxicity and worsen diseases.	Determination of optimal concentrations for efficacy without phytotoxicity.	(Bordoh *et al*., 2020)
Apple	Neem (*Azadirachta indica* A. Juss.), fennel, lavender, thyme, pennyroyal (*Mentha pulegium* L.), salvia (*Salvia officinalis* L.), and asafetida (*Ferula assafoetida* L.) extracts	Neem extract (25 %) reduced disease severity by 89.11 % against *Botrytis cinerea*.	Variability in effectiveness among different extracts.	(Gholamnezhad, 2019)

PHYSICAL AND ENVIRONMENTAL PRESERVATION METHODS

Controlled atmosphere storage

Controlled atmosphere storage (CAS) was developed as a complement to refrigeration and is widely used for a variety of fruits and vegetables (Fang and Wakisaka, 2021). Among postharvest storage techniques, CAS is a popular choice. This method involves maintaining optimal gas concentrations by continuously adding or removing gases during the storage of unpackaged produce (Kirtil and Oztop, 2016). By modifying the surrounding atmosphere, CAS alters the internal gaseous composition of the product, which in turn affects cell metabolism. Most research on CAS in fruit and vegetables involves combining it with other technologies, such as modified atmosphere packaging (MAP), 1-methylcyclopropene (1-MCP), high-pressure carbon dioxide (H-CO_2), argon-modified atmosphere packaging (MAP-Ar), and MAP with zinc oxide (ZnO), among others (Fang and Wakisaka, 2021) (Table 2). Combined with cold storage, CAS reduces metabolic activity, inhibits enzymatic reactions, and effectively extends the shelf life of fruits and vegetables (Falagán and Terry, 2018).

Table 2. Recent research on controlled atmosphere storage of fruit and vegetables.

Product	Treatment	Storage time	Results	Limitations/notes	References
Apple (*Malus domestica* L.)	1.2 kPa O_2, 2 kPa CO_2 94 ± 2 % relative humidity (RH), 1.5 ± 0.1 °C	240 d	Reduced ester production and volatile profile quality.	May affect flavor and aroma profile of apples.	(Anese *et al*., 2020)
Apple	2–3 % O_2, 1–1.5 % CO_2 90–95 % RH, 0 ± 0.5 °C,	210 d	Improved quality and prevented pericarp browning.	Potential variability in response depending on apple variety.	(Gao *et al*., 2023)
Apple	<2 % O_2, <1 % CO_2 99 % RH, 1.5–2 °C	>150 d	Preserved phenolic compounds for long-term storage.	Requires precise control of gas levels to avoid quality deterioration.	(Bílková *et al*., 2020)
Pear (*Pyrus communis* L.)	1.0 % O_2 and 2.2 % O_2, ~0.05 % CO_2	8-10 months	Quality improved by 2.2 % O_2 for pears harvested at 48.01 N firmness, and by 1 % O_2 for those at 42.19 N firmness or lower.	Effectiveness of O_2 concentration varies with harvest firmness.	(Dong *et al*., 2023b)
Grape (*Vitis vinifera* L.)	6 % O_2, 10 % CO_2 95 % RH, 4 °C	42 d	Decreased weight loss, decay incidence, and ethylene production; maintained antioxidant activity, phenolic compounds, and firmness.	Requires careful monitoring to avoid excessive CO_2 damage.	(Shahkoomahally *et al*., 2021)

Table 2. Continued.

Product	Treatment	Storage time	Results	Limitations/notes	References
Dragon Fruit (*Selenicereus undatus* (Haw.) D.R. Hunt)	2 kPa O_2, 5 kPa CO_2 6 ± 0.5 °C	50 d	Maintained acidity and reduced scale yellowing.	Optimal conditions may vary with different dragon fruit varieties.	(Ho *et al*., 2021)
Cabbage (*Brassica oleracea* L.)	2 % O_2, 5 % CO_2 93 % RH, 0 °C	150 d	Reduced weight loss, trimming loss, and decay incidence; maintained pH, soluble solids, moisture, and reduced sugar content.	Long storage times require stringent control of environmental conditions.	(Choi *et al*., 2020)
Cabbage	2 % O_2, 2 % CO_2 95 % RH, 2 °C	100 d	Maintained vitamin C, total phenolics, and total glucosinolates; significantly inhibited total aerobic bacterial growth compared to control.	Requires optimization for different cabbage cultivars and storage conditions.	(Kang *et al*., 2019)
Beans (*Phaseolus vulgaris* L.)	1.5 kPa O_2, 9 kPa CO_2 40–60 % RH, 20 °C	180 d	Observed changes in galactose, starch, and sucrose pathways; no changes in amino acid or lipid metabolism.	Potential for physiological changes affecting long-term quality.	(Coelho *et al*., 2020)
Pecan nuts (*Carya illinoinensis* (Wangenh.) K.Koch)	2 kPa O_2 combined or not with CO_2 40 or 80 kPa 10 °C	12 months	Higher luminosity, lower acidity, TBARS, and peroxide than control; no significant differences were observed between 40 and 80 kPa CO_2.	Optimal CO_2 concentration requires further study for different nut varieties.	(Ribeiro *et al*., 2023)
Figs (*Ficus carica* L.)	3 % O_2, 15 % CO_2 90–95 % RH, 0 °C	28 d	Decreased respiration and decay rates; higher sugar and organic acid content compared to control.	Requires precise control of gas composition to avoid flavor and texture degradation.	(Dogan and Erkan, 2023)

To ensure the effectiveness of CAS, several factors must be taken into account, including the type of product (fruit species and cultivar), its climacteric behavior, storage temperature, stage of maturity, quality at harvest, pre-storage treatments, and gas concentrations (Valdez-Fragoso and Mújica-Paz, 2016). Like any postharvest technology, CAS can cause tissue damage under certain conditions, such as excessively low oxygen or excessively high carbon dioxide concentrations. When oxygen levels are too low, anaerobic respiration and fermentation are induced, leading to the formation of off-flavors and toxic compounds such as alcohols and aldehydes, which can ultimately cause tissue death. This process typically begins in the central part of the fruit (Ntsoane *et al*., 2019). Conversely, excessively high carbon dioxide levels can produce damage similar to chilling injury, including internal and surface browning, pitting, and tissue softening (DeEll *et al*., 2016).

Modified atmosphere packaging

The respiratory behavior of fresh postharvest commodities and the subsequent development of ripening and senescence are influenced by temperature, the atmosphere that surrounds the product during storage, and the concentration of gases that have a high effect on its metabolism, such as ethylene, water vapor, O_2, and CO_2 (Castellanos and Herrera, 2017). By regulating temperature and the gaseous composition of the atmosphere surrounding fruits and vegetables, it is possible to slow biochemical processes and thereby extend shelf life. Likewise, the growth and activity of microorganisms are strongly influenced by environmental conditions and can be controlled by modifying these parameters (del Valle *et al*., 2009; Sandhya, 2010; Caleb *et al*., 2012).

In modified atmosphere packaging (MAP), the food is packaged, and the gas composition inside is modified to specifically extend the product's shelf life. This modification can be achieved in two ways: in active MAP, the gas mixture is adjusted at the beginning of the packaging process, whereas in passive MAP, the internal atmosphere is altered over time through the natural respiration of the product and the selective permeability of the packaging film (Kirtil and Oztop, 2016).

Organoleptic characteristics are important in consumer purchasing decisions, and storage or packaging under modified atmospheres can significantly influence these attributes. (Thompson, 2015). During food distribution, temperature fluctuations commonly occur, and a modified atmosphere in packaged food can lead to excessive O_2 depletion and CO_2 accumulation, leading to metabolic disorders and reduced shelf life (Rodriguez-Aguilera *et al*., 2009). Adjusting moisture content, together with temperature control and a specific atmospheric composition, has proven effective in maintaining the quality of fruits and vegetables and extending their shelf life (Falagán and Terry, 2018).

Gas absorbents and release agents are added to the packaging bag to remove excessive ethylene, CO_2, and moisture. This allows for timely replenishment of O_2, maintaining a suitable gas environment for preservation. These technologies, known as active packaging, commonly incorporate antibacterial and deoxidizing agents such as chitosan and nisin. However, these chemicals may contaminate food during long-term storage, posing a risk to consumers' health (Chang *et al*., 2023). Active packaging can preserve the quality of food for an extended period, delay or prevent oxidative, microbial, and enzymatic spoilage, reduce contamination and weight loss, and preserve the color and texture during storage (Park *et al*., 2018).

When designing a MAP system, it is important to consider factors affecting both the metabolism of the produce and the permeability of the packaging to gases. These factors include the type of produce and its ripening stage, storage conditions, and specific characteristics of the packaging system. For the packaged product, it is important to determine its transpiration rate, oxygen consumption, and CO_2 and ethylene generation rates under the given packaging conditions. Additionally, identifying the optimal gas concentrations and relative humidity levels that enhance shelf life is necessary (Castellanos and Herrera, 2017) (Table 3).

Table 3. Recent research on modified atmosphere packaging (MAP) for fruit and vegetables: efficacy and limitations.

Product	Treatment	Storage time	Results	Limitations/Notes	References
Tomato (*Solanum lycopersicum* L.)	14.9–16.7 % O_2, 4.2–7.3 % CO_2 95 %, 4 °C	14 + 3–8 d at 20 °C	Reduced chilling injury and extended shelf life.	Temperature fluctuations can affect effectiveness.	(Park *et al*., 2018)
Mulberry (*Morus alba* L.)	10 % O_2, 5 % CO_2, 85 % Ar 90 ± 5 % relative humidity (RH), 4 ± 1 °C	12 d	Reduced weight loss, maintained juice content, and resulted in higher soluble solids content, but had no effect on sensory profile.	Requires precise control of gas composition.	(Tinebra *et al*., 2021)
Strawberry (*Fragaria × ananassa* Duch.)	5–10 % O_2, 10–15 % CO_2 90–95 % RH, 4 ± 1 °C	18 d	Decelerated chemical spoilage and controlled microbial spoilage.	Sensitivity to CO_2 levels may vary among different strawberry varieties.	(Kahramanoğlu, 2019)
Jujube (*Ziziphus jujuba* Lam.)	25 % O_2, 5 % CO_2 85 ± 5 % RH, 2 ± 1 °C	9 + 35 d of cold storage	Ineffective in maintaining weight and firmness, or reducing browning.	Not suitable for long-term storage without additional treatments.	(Moradinezhad and Dorostkar, 2021)
Pistachio (*Pistacia vera* L.)	10 % O_2, 90 % N_2 85–90 % RH, 5 °C	45 d	Reduced weight loss and aflatoxin production, and effectively controlled hull color deterioration.	Potential for uneven gas distribution within packaging.	(Rezaiyan Attar *et al*., 2023)
Lettuce (*Lactuca sativa* L.)	97 % N_2 and 3 % O_2. CO_2 90 % RH, 4 °C	20 d	Reduced respiration, transpiration, water loss, and deterioration.	Needs consistent temperature control to avoid spoilage.	(Soltani Firouz *et al*., 2021)
Tomato	10 % O_2, 10 % CO_2 91 % RH, 6 °C	21 d	Retarded color development, firmness loss, and decay.	Varietal differences may impact effectiveness.	(Olveira-Bouzas *et al*., 2021)
Mango (*Mangifera indica* L.)	7 % CO_2, 3 % O_2, 90 % N_2 13 ± 1 °C	30 d	Delayed pulp yellowing and biochemical changes, and maintained firmness and free moisture content.	High CO_2 concentrations can cause off-flavors.	(Wei *et al*., 2021)

Table 3. Continued.

Product	Treatment	Storage time	Results	Limitations/Notes	References
Wolfberry (*Lycium barbarum* L.)	5 % O_2, 10 % CO_2 0 ± 0.5 °C	28 d	Maintained physiological quality, and reduced weight loss, decay index, and color change.	Optimal conditions need to be determined for different storage durations.	(Liang *et al*., 2022)
Apricot (*Prunus armeniaca* L.)	40 % O_2, 20 % CO_2	6 + 28 d of cold storage	Decreased weight loss and total soluble solids, maintained hardness and chemical properties, but caused no signs of decay.	High oxygen levels may increase respiration rate.	(Dorostkar *et al*., 2022)
Lychee (*Litchi chinensis* Sonn.)	5 % O_2, 20 % CO_2, 75 % N_2 90 ± 5 % RH, 5 ± 1 °C	9 d	The treatment prevented browning, and maintained the pericarp color, vitamin content, flavor, and microbiological quality.	Prevented browning, maintained pericarp color, vitamin content, flavor, and microbiological quality.	(Passafiume *et al*., 2023)
Sweet cherry (*Prunus avium* L.)	5 % O_2, 10 % CO_2 80–85 % RH, -1 to +1 °C	120 d	Decreased decay rate, reduced polyphenol oxidase and peroxidase activity, and maintained firmness, nutrition, and taste.	Requires precise temperature control to prevent freeze damage.	(Xing *et al*., 2020)

Irradiation

Irradiation is a safe method in which food is exposed to radiation for a specific period to eliminate pathogenic microorganisms, parasites, and fungi. This technology helps prevent sprouting, delay ripening, eliminate insects, and sterilize products. Some advantages are its superior effectiveness to fungicides in the inactivation of pathogens and minimal or negligible sensory changes in the product. In addition, irradiation does not leave chemical residues in the product or make it radioactive and allows sterilization without contact. However, although irradiation is considered an energy-efficient and safe process for the consumer and the environment, there is still low acceptance of irradiated foods due to lack of consumer awareness. Other disadvantages include high capital costs, localized radiation hazards, and taste changes due to oxidation of lipids in the food (Jeong and Jeong, 2017; Bhatnagar *et al*., 2022).

Radiation can be either ionizing or non-ionizing, depending on the intensity of the energy transmitted. Non-ionizing radiation is a type of energy that can travel in space in the form of electromagnetic waves at a certain wavelength. This radiation comprises a portion of the electromagnetic spectrum from 1 to 3×10^{15} Hz, with a long wavelength (>100 nm) and low photon energy (<12.4 eV). It is only able to stimulate one molecule or atomic electron and is therefore not normally dangerous. Some sources of non-ionizing radiation are extremely low-frequency electromagnetic fields, radiofrequency, infrared, microwave, and ultraviolet, the latter being one of the most investigated radiations in postharvest of fruits and vegetables (Bisht *et al*., 2021).

Ultraviolet (UV) radiation lies outside the visible light spectrum and is classified as highly energetic non-ionizing radiation. It can be absorbed by microorganisms, where it induces biochemical alterations in enzymes or nucleic acids or activates specific molecules that form phototoxins, disrupting physiological processes. The effectiveness of UV radiation ranges from 89 to 99.999% against bacteria, fungi, and viruses, depending on factors such as microorganism type, radiation dose, exposure duration, and environmental conditions (Jalbout and Dgheim, 2019; Khan *et al*., 2022). Based on wavelength, UV radiation is divided into three regions: UV-C (200–280 nm, shortwave), UV-B (280–320 nm, medium wave), and UV-A (320–400 nm, longwave). In postharvest applications, UV-C treatment typically involves exposing produce to UV-C lamps emitting at 254 nm, the wavelength most effective in damaging microbial DNA. However, excessive exposure can compromise product quality, leading to surface damage such as browning or scorching (Murray *et al*., 2019).

Ionizing radiation, in contrast, possesses high energy, capable of removing electrons from atoms, thereby forming ions. This type of radiation is characterized by very short wavelengths and includes X-rays, gamma rays, and electron beams (Bisht *et al*., 2021). Ionizing radiation impacts microorganisms and food products with direct and indirect effects. The direct effect involves damage to cellular components, such as carbohydrates, lipids, and DNA. The indirect effect involves free radicals and reactive species, including hydroxyl radicals, hydrogen atoms, and hydroelectrons from water radiolysis, that react with cells and food components (Bhatnagar *et al*., 2022).

Several studies have demonstrated the effectiveness of irradiation as a postharvest treatment for food. UV-C radiation doses of 0.6–6 kJ m^{-2} reduced 2.2–3.1 and 2.3–3.5 log CFU per fruit, respectively, for cocktail mixtures of *Salmonella enterica* and *E. coli* O157:H7 on the surface of tomato (Mukhopadhyay *et al*., 2014). Similarly, strawberries treated with gamma irradiation at 1 and 1.5 kGy exhibited significantly extended storage lives (5.75 and 7.75 d, respectively) compared with non-irradiated controls (3.25 d), without altering key chemical quality parameters such as soluble solids, titratable acidity, or pH (Majeed *et al*., 2014). In the case of X-rays, exposure of whole melons to a 2 kGy dose achieved a 5-log CFU reduction across all tested pathogens (*E. coli* O157:H7, *L. monocytogenes*, *S. enterica*, and *Shigella flexneri*) without affecting the color or firmness of the fruits (Mahmoud, 2012).

Cold plasma

Plasma is an electrically neutral gas containing numerous ionized particles and can be artificially generated through energy sources such as electrical discharges, radio frequencies, or microwaves (Ekezie *et al*., 2017). Among novel postharvest technologies, cold plasma has shown high efficacy in sterilizing fruits and vegetables while maintaining or even improving their quality under certain conditions (Chen *et* *al*., 2020). Most studies have reported minimal or no impact on the physical, chemical, nutritional, and sensory attributes of different products, giving it a clear advantage over conventional heat treatments. Moreover, cold plasma is an affordable and eco-friendly technology (Pankaj *et al*., 2018).

Cold plasma was effective in inactivating microorganisms in blueberries but affected color and anthocyanins in treatments with longer exposure time (Lacombe *et al*., 2015). In strawberries, cold plasma caused a reduction in aerobic mesophilic bacteria, molds, and yeasts, while firmness, color, and respiration rate were not affected (Misra *et al*., 2014). Similarly, in cherry tomatoes, there was no effect on color, firmness, pH, and total soluble solids with cold plasma application, but there was a significant reduction in *E. coli* and *L. innocua*, as well as a decrease in mesophilic bacteria, molds, and yeasts (Ziuzina *et al*., 2016).

EDIBLE COATINGS AND FILMS

An edible coating is defined as a continuous matrix composed of edible and food-safe compounds that forms directly around the food and can be safely consumed by the consumer. These coatings are typically applied by dipping or spraying the product. In contrast, edible films or biofilms are thin layers usually pre-formed on plates or molds (casting method) or by extrusion, serving as the primary protective envelope for the food. Edible coatings and films extend the shelf life of foods by forming a semi-permeable barrier to water vapor and gases, which reduces weight loss and slows metabolic activity in fruits and vegetables. Additionally, these formulations can incorporate active substances, such as antioxidants and natural antimicrobial agents, helping to preserve food quality and safety throughout transport, marketing, and consumption (Pérez-Gago and Palou, 2016).

The main ingredients used in edible coating formulations are proteins (casein, soy protein, whey protein), polysaccharides (cellulose and its derivatives, starch, pectin, carrageenans, gums, chitosan), and lipids (fatty acids, mono- and diglycerides of fatty acids, natural waxes), either alone or in combinations (Bautista-Baños *et al*., 2017). In addition, certain food additives, such as plasticizers, emulsifiers, and active substances such as antioxidants, vitamins, and antimicrobial agents, can be added to formulations to improve the physical properties of formulations and enhance the microbiological, nutritional, and organoleptic properties of foods (Ansorena *et al*., 2018). The use of natural antimicrobials in both coatings and edible films, such as essential oils, chitosan, herbal extracts, plants, and spices, has been extensively studied for controlling microorganisms in food (Irkin and Esmer, 2015; Etxabide *et al*., 2017).

CONCLUSIONS

The deterioration of fruits and vegetables during the postharvest period depends on several physiological and environmental factors. The development of treatments and technologies complementary to refrigeration can prevent or delay physicochemical and microbiological deterioration. Ozone and electrolyzed water stand out for their effectiveness in eliminating microbes while remaining safe for consumers. Modified atmosphere packaging and edible coatings primarily preserve physicochemical and sensory quality, and when enriched with antimicrobial agents, they also enhance microbiological safety. Cold plasma has emerged as a promising technology, demonstrating strong activity against pathogenic bacteria and fungi with no negative impact on product quality. The effectiveness of any given approach depends on the fruit species and cultivar, its physiological condition at harvest, and storage conditions. Consequently, targeted studies are essential, particularly to assess the potential of emerging technologies for extending the shelf life of fresh produce.

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Agrociencia

RHIZOSPHERIC BACTERIAL COMMUNITIES AND SEEDLING GROWTH OF *Fraxinus uhdei* Wenz. Lingelsh.

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ABSTRACT

The application of plant growth-promoting rhizobacteria (PGPR) induces shifts in rhizospheric bacterial community composition. This study evaluated the effects of four *Azospirillum brasilense* strains in the rhizosphere of ash tree (*Fraxinus uhdei* Wenz. Lingelsh.) seedlings: two diazotrophic (nitrogen (N_2) fixing) and two phosphate solubilizing strains. The analysis encompassed bacterial community structure, potential metabolic functions, and seedling growth and nutrition responses. DNA was extracted from rhizospheric soil, and the V3–V4 hypervariable region of the 16S rRNA gene was amplified and sequenced. The rhizobiome of *A. brasilense* inoculated plants was predominantly composed of seven phyla, with Proteobacteria being the most abundant (54 %). Alpha diversity analysis assessed by the Chao 1 richness and Shannon Weaver indices indicated that inoculation did not significantly alter species richness but resulted in a reduction of diversity. Inoculation with nitrogen-fixing (NF) strains led to a sevenfold increase in the relative abundance of the genus *Azospirillum* compared to uninoculated plants (Psi). Beta diversity analysis based on Bray-Curtis dissimilarity revealed a significant separation between the bacterial communities of inoculated and control plants. Prediction of metabolic potential via the Kyoto Encyclopedia of Genes and Genomes (KEGG) indicated that inoculation significantly altered ($p \leq 0.05$) the relative abundance of 11 % of the predicted potential functional pathways. Analysis of variance (ANOVA) and the post-hoc Tukey test ($p \leq 0.05$) demonstrated that co-inoculation with both strains yielded the most significant growth enhancements, with increases of 11.4 to 36.9 % across measured variables. FN strains increased macronutrient content by 15 to 24 %. These findings demonstrate that *A. brasilense* inoculation influences the structure and predicted functions of the rhizobacterial community and acts as an effective PGPR by enhancing the growth and nutrition of *F. uhdei* seedlings.

Keywords: *Azospirillum brasilense*, rhizosphere, 16S rRNA.

INTRODUCTION

With the advancement of omics sciences in the last decade, the perception of plants as a single individual has changed. They are no longer considered isolated organisms but rather holobionts, that is, larger entities made up of a host (plant) and its associated microorganisms (Cook *et al*., 2024). These phyllospheric, endophytic, and rhizospheric microorganisms affect plant development and growth. In the rhizosphere, which is the area surrounding the roots, bacteria have gained special interest due to their role in nutrient supply and the production of phytohormones and antimicrobial substances. For this reason, they are called plant growth-promoting rhizobacteria (PGPR) (Dutta *et al*., 2022).

In tree species, the composition of rhizosphere bacterial communities has been described during nursery plant growth. The core microbiome is composed of nitrogen-fixing (N_2) and plant growth-promoting taxa (García-Lemos *et al*., 2020). Bacterial groups involved in plant-microorganism interactions, which participate in plant metabolism and nutrition, have also been identified (Liu *et al*., 2022).

The diversity and composition of bacterial communities can be altered by agricultural practices such as the use of biofertilizers. In *Ulmus chenmoui* Cheng, the introduction of phosphate-dissolving bacteria changes the composition of the bacterial community. This benefits populations of Proteobacteria and Bacteroidetes, which are part of the carbon and nitrogen cycles (Song *et al*., 2021). These findings highlight the importance of evaluating the effects of PGPR biofertilizers on the diversity and composition of the bacterial microbiome, given that they can influence the formation of communities that favor plant growth (Gu *et al*., 2020).

The ash tree (*Fraxinus uhdei* Wenz. Lingelsh.) is commonly found in urban areas of Mexico. Its establishment is widely favored in parks and avenues, as it can survive in poorly developed and contaminated soils (Pérez-Baltazar *et al*., 2020). However, *F. uhdei* plants intended for urban tree planting could enhance their growth and nutrient availability with the use of PGPR-based biofertilizers.

The objective of this study was to determine whether inoculation with *Azospirillum brasilense*, a PGPR, using two strains capable of fixing nitrogen (N_2) and two strains capable of solubilizing phosphates, impacts the diversity, composition, and potential metabolic functions of the bacterial community in the rhizosphere of *F. uhdei* seedlings. The effect on their growth and nutrition was also evaluated. The null hypothesis states that inoculation with *A. brasilense*, either with one or both strain types, causes no changes in the structure of the rhizospheric bacterial community and does not favor plant growth and nutrition.

MATERIALS AND METHODS

Site location and experimental conditions

The trial was established from January 23rd to August 15th, 2024, in Texcoco, State of Mexico (19° 29′ 23.74″ N, 98° 53′ 4.09″ W, at an altitude of 2250 m), under greenhouse conditions, covered with 50 % shade netting. There was no artificial control of temperature and humidity. The average, minimum, and maximum temperature values were 17.2, 3.8, and 30.1 °C, respectively, while relative humidity was 47.2, 12.2, and 81.4 %. These records were obtained with an RC-4HC datalogger thermo-hygrometer (Elitech, Mexico) with a storage capacity of 16 000 readings, programmed to take measurements every hour.

The substrate used for sowing, production, and inoculation of *F. uhdei* plants consisted of a mixture of Sunshine® Mix N3 peat, perlite, and vermiculite in a 60:30:10 (v/v) ratio. The substrate was sterilized three times with water vapor for 5 h. Multicote® 18-6-12 (N-P-K) controlled-release fertilizer was used as a nutrient source. According to the manufacturer, nutrients are released within 8–9 months at a constant temperature of 21°C.

Biological material

The *A. brasilense* inoculant with N_2-fixing strains was prepared in a sterile, semi-solid nitrogen-salt malate (Nfb) medium supplemented with malic acid, mineral salts, vitamins, and a nitrogen source. Incubation was carried out at 32 °C, with shaking at 220 rpm for 48 h. The bacterial concentration achieved was 5×10^8 colony-forming units per milliliter (CFU L^{-1}). In addition to their N_2-fixing capacity, these strains produce indoleacetic acid (IAA). Molecular identification and biochemical characterization were performed according to Carcaño-Montiel *et al*. (2006).

The *A. brasilense* inoculant with phosphate-solubilizing and IAA-producing strains was prepared in nutrient broth or trypticase soy broth. Incubation was carried out at 32 °C, shaking at 180 rpm for 24 h, until a population density of 8 × 108 CFU mL^{-1} was reached. Information on the molecular and biochemical characterization of these strains is not available due to their confidentiality (registration number 1160672, Mexican Institute of Industrial Property). Both inoculum sources belong to and were provided by the Soil Microbiology Laboratory of the Center for Research in Microbiological Sciences at the Meritorious Autonomous University of Puebla, Mexico.

The *F. uhdei* seeds were collected from adult trees located in the "El Ranchito" experimental field at the Autonomous University of Chapingo, in the municipality of Texcoco, State of Mexico, Mexico. They were disinfested with 30 % H_2O_2 for 20 min. They were subsequently rinsed three times with sterile distilled water. Sowing was carried out in 380 cm^3 polypropylene forest tubes, ensuring the growth of one seedling per tube by placing two seeds in each. At 26 d after sowing, the germination rate was 76 %. Irrigation was provided every third day with potable water. Inoculation was carried out at planting, adding 10 mL of inoculum to each tube. A second inoculation was carried out 90 d after planting, adding 15 mL of inoculum per plant.

Sample collection for bacterial communities

Samples were obtained from six-month-old plants grown with a fertilizer dose of 4 g L^{-1} of substrate. Under aseptic conditions for instruments and consumables, soil firmly adhered to fine roots was obtained, covering the surface, center, and bottom of the root ball. In 2 mL Eppendorf ONiLAB® conical-base tubes, 250 mg of soil were placed for each plant, adding 400 μL of DNA/RNA Shield protective reagent (Zymo Research, USA). The samples were kept at -20 °C until further laboratory analysis.

DNA extraction and sequencing of the 16S rRNA gene

Substrate sterilization with water vapor or other methods, such as cobalt-60 soil irradiation, does not prevent the rapid reconstruction of microbial communities from diverse environmental sources (Ferrarezi *et al*., 2023). Therefore, rhizosphere soil samples were sent to BGI Innomics Inc. (Tai Po, Hong Kong) for DNA extraction and amplicon sequencing. DNA was extracted using the QIAGEN DNeasy PowerSoil Pro Kit (Hilden, Germany) isolation kit, following the manufacturer's extraction protocol. DNA concentration and purity were assessed by fluorescence in a microplate reader. Amplicons were obtained from the V3–V4 variable region of the 16S rRNA gene by polymerase chain reaction (PCR), using the primer pair 341F (ACTCCTACGGGAGGCAGCAG) and 806R (GGACTACHVGGGTWTCTAAT), and sequenced using the DNBSEQ platform (Hu *et al*., 2024).

Sequencing data processing

Sequencing data were analyzed using USEARCH v7.0.1090 (Farooq *et al*., 2024) and filtered to obtain high-quality, clean readings. Readings with low-quality base pairs (20–25 base pairs less than the average) were truncated, and those that after truncation retained 75 % or less of their original length were eliminated. In addition, readings contaminated with adapter sequences (allowing up to three bases of discrepancy), ambiguous readings (N), and low-complexity readings (10 consecutive repeated bases) were discarded.

Chimera sequences were subsequently removed using the UCHIME algorithm. Genetic barcodes, used to align readings and ensure accurate assignment to the corresponding sample (zero-base mismatch), were also removed. The sequences were grouped into operational taxonomic units (OTUs) with a similarity level of 97 %.

The alpha diversity analysis of the bacterial communities was performed using the Mothur software v.1.31.2 (Schloss, 2020), which was used to obtain rarefaction curves and estimate the Chao 1 richness and Shannon Weaver alpha diversity indices. For the beta diversity analysis, QIIME v1.8.0 (Ibarra-Sánchez and Romero-Salas, 2024) was used. The similarity and dissimilarity of the bacterial communities was visualized using the principal coordinates analysis (PCoA) with the Bray-Curtis dissimilarity test. To evaluate differences between groups, the non-parametric statistical test of similarity analysis (ANOSIM) was performed.

Potential metabolic functions were predicted from the Kyoto Encyclopedia of Genes and Genomes (KEGG) using PICRUSt2 v2.3.0-b (Douglas *et al*., 2019). Amplicon sequence variants (ASVs) were obtained using the DADA2 algorithm. ASVs were inserted into a reference phylogenetic tree obtained from prokaryotic genomes available in the Integrated Microbial Genomes system (https://img.jgi.doe.gov/) to determine their most likely phylogenetic position. Subsequently, hidden state prediction (HSP) was performed using the Castor algorithm based on the evolutionary relationship of the ASVs to microorganisms with known genome-wide sequences. The predicted functional genes, identified from KO identifiers (KEGG Orthology), were grouped according to the functional hierarchy provided by the KEGG database. Finally, potential metabolic functions at level three (specific metabolic pathways) were obtained, and their relative abundance in each sample was determined.

Morphological variables

The growth of *F. uhdei* seedlings was evaluated based on their total height, stem diameter at the root collar, leaf area, root and above-ground biomass, and total dry biomass. Total height was measured with a measuring tape, starting at the root collar and ending at the apex of each plant. Stem diameter was measured with a KEATRONIC digital caliper. Leaf area was obtained with a leaf area integrator (LI-300, LI-COR; Lincoln, NE, USA). Biomass was determined by drying the above-ground and root components for 3 d at 70 °C in a forced-air oven (Riossa, HCF-125D, Mexico). The material was weighed on an analytical balance (OHAUS model E01140, NJ, USA). Leaf area and biomass were measured from four randomly selected plants per treatment. Total height and root collar diameter were recorded for all experimental units.

Nutritional analysis

At the end of the trial (206 d), four plants per treatment were randomly selected for nutrient analysis. Atomic absorption spectrometry was used to determine the P, K, Ca, and Mg contents based on the methods described by Alcántar-González and Sandoval-Villa (1999). Nitrogen content was obtained by the micro-Kjeldahl method (Bremner, 1965).

Experimental design and statistical analysis

The assay for analysis of bacterial communities was established under a completely randomized experimental design. The established treatments were: 1) inoculation with N_2-fixing *A. brasilense*, 2) simultaneous inoculation with N_2-fixing *A. brasilense* and phosphate-solubilizing *A. brasilense*, and 3) control without inoculation. For each treatment, four *F. uhdei* plants (biological replicates) were randomly selected from a total of 20 plants per treatment.

The plant growth and nutrition trial was conducted using a completely randomized, factorial design, with two doses of controlled-release fertilizer and four inocula (2 × 4). Fertilization levels were established with the application of 2 and 4 g L^{-1} of substrate. The inoculum sources were: N_2-fixing *A. brasilense* (FN), phosphate-solubilizing *A. brasilense* (SF), the simultaneous combination of both (*A. brasilense* FN+SF), and a non-inoculated control (Psi). The combination of factors and levels resulted in eight treatments. Each treatment was replicated 20 times, considering one plant as the experimental unit for a total of 160 plants.

Bacterial community results were analyzed using the Kruskal-Wallis test. Analysis of variance (ANOVA) was used for growth and nutrient analysis after verifying normality assumptions (Shapiro-Wilk test). When treatment effects were present, means were compared using Tukey's honest significant difference test with a significance level of $\alpha = 0.05$. Statistical analysis was performed using the R statistical package (R Core Team, 2024).

RESULTS AND DISCUSSION

Composition of bacterial communities

The bacterial community composition of the three inoculation groups generated 2969 bacterial OTUs (1601 ± 18.8 per sample). The OTUs were assigned to a single kingdom, 33 phyla, 62 classes, 110 orders, 203 families, 418 genera, and 443 species. Seven bacterial phyla accounted for 86.4 % of the relative abundance; of these, *Candidatus* Saccharimonadota was significantly affected in abundance ($p \leq 0.05$) by the FN+SF strain combination (Figure 1A). This phylum is distributed in diverse environments; in the rhizosphere of *Larix decidua* Mill, it constitutes up to 10 % of the total relative abundance. However, knowledge of its functions is still limited (Praeg and Illmer, 2020).

The bacterial community was dominated by the phyla Proteobacteria (54.7 ± 0.7 %) and Bacteroidota (14 ± 2 %). The genera exhibiting the highest relative abundance collectively accounted for 18.8 % of the total community (Figure 1B). These findings contrast with prior studies on forest soils. For instance, Proteobacteria was reported at a lower relative abundance (35.3 to 37 %) in the root system soil of various tree genera, including *Fraxinus, Pinus, Larix, Quercus, Fagus,* and *Carpinus* (Staszel-Szlachta *et al*., 2024). A community composition more similar to the one observed here was reported in the study of *Alnus nepalensis* D. Don, where Proteobacteria (37.2 %) and Bacteroidota (12 %) were also the dominant phyla (Sen *et al*., 2022). These phyla were associated with nitrogen-fixing bacterial taxa, which were enriched in the rhizospheric soil. Approximately 50 % of the bacterial community in the rhizosphere possessed N_2 fixation mechanisms (Sen *et al*., 2022).

The increased presence of NF taxa in the rhizosphere of *F. uhdei* could be enhanced by inoculation with NF bacterium *A. brasilense*, which showed a significant effect ($p \leq 0.05$) by increasing the abundance of the genus *Azospirillum* by 7- and 4-fold compared to Psi and FN+SF, respectively (Figure 1B). The genus *Ohtaekwangia,* belonging to Bacteroidota, decreased 1- and 2-fold in FN and FN+SF. This genus has been identified in the rhizosphere of agricultural crops such as olive (*Olea europaea* L.) and is considered a biomarker of the optimal state of soil amendments (Palla *et al*., 2022). The influence

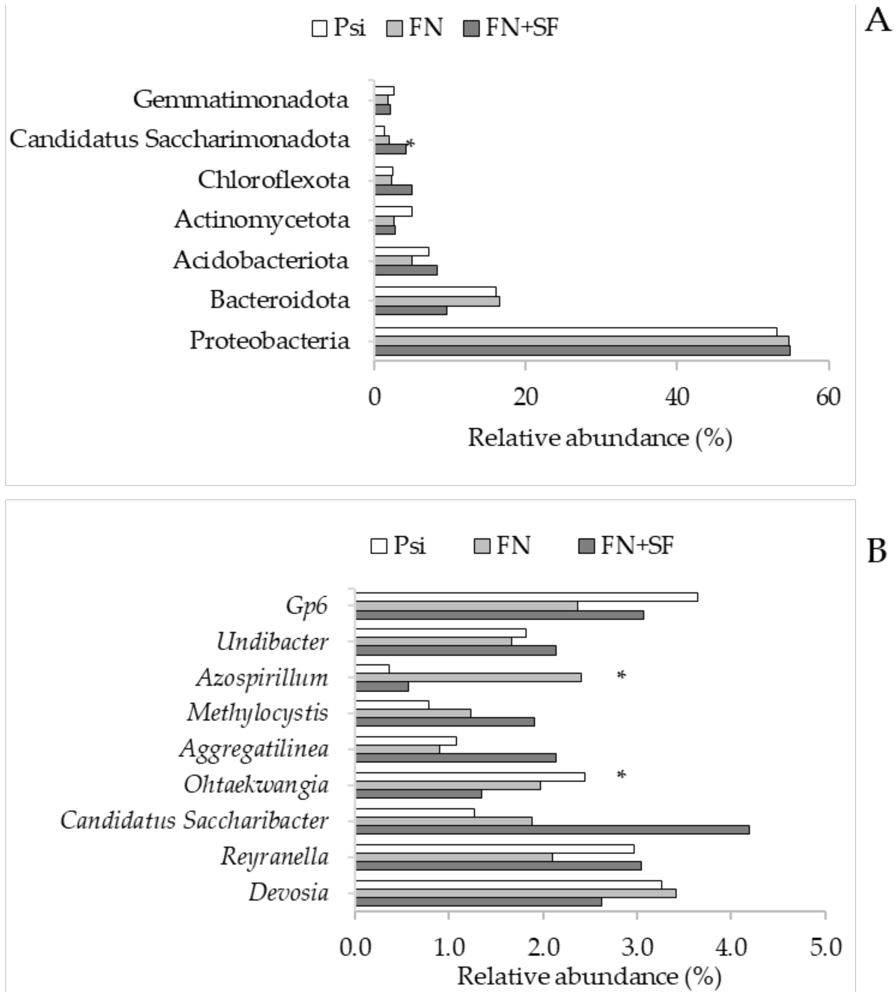

Figure 1. Composition of bacterial communities associated with the rhizosphere of *Fraxinus uhdei* Wenz. Lingelsh. A: Bacterial phyla with the highest relative abundance (%); B: Bacterial genera with the highest relative abundance (%). Psi: Uninoculated plants; FN: plants inoculated with N_2-fixing *Azospirillum brasilense*; FN+SF: plants inoculated with N_2-fixing *A. brasilense* and phosphate-solubilizing *A. brasilense*.

of inoculation treatments on a single phylum and bacterial genus indicates that the rhizospheric microbiome of *F. uhdei* shows minimal changes due to the use of PGPR.

Richness and diversity of the bacterial rhizosphere

Rarefaction curves based on the coverage index were stable from 20 000 to 60 000 sequences (close to 1.0), suggesting sufficient sampling depth to capture most of the bacterial diversity (Figure 2A). The overlap of the rarefaction curves indicates that the rhizospheric bacterial microbiome of *F. uhdei* does not show volatility upon PGPR inoculation. The Chao 1 estimator based on rare species counts showed that bacterial

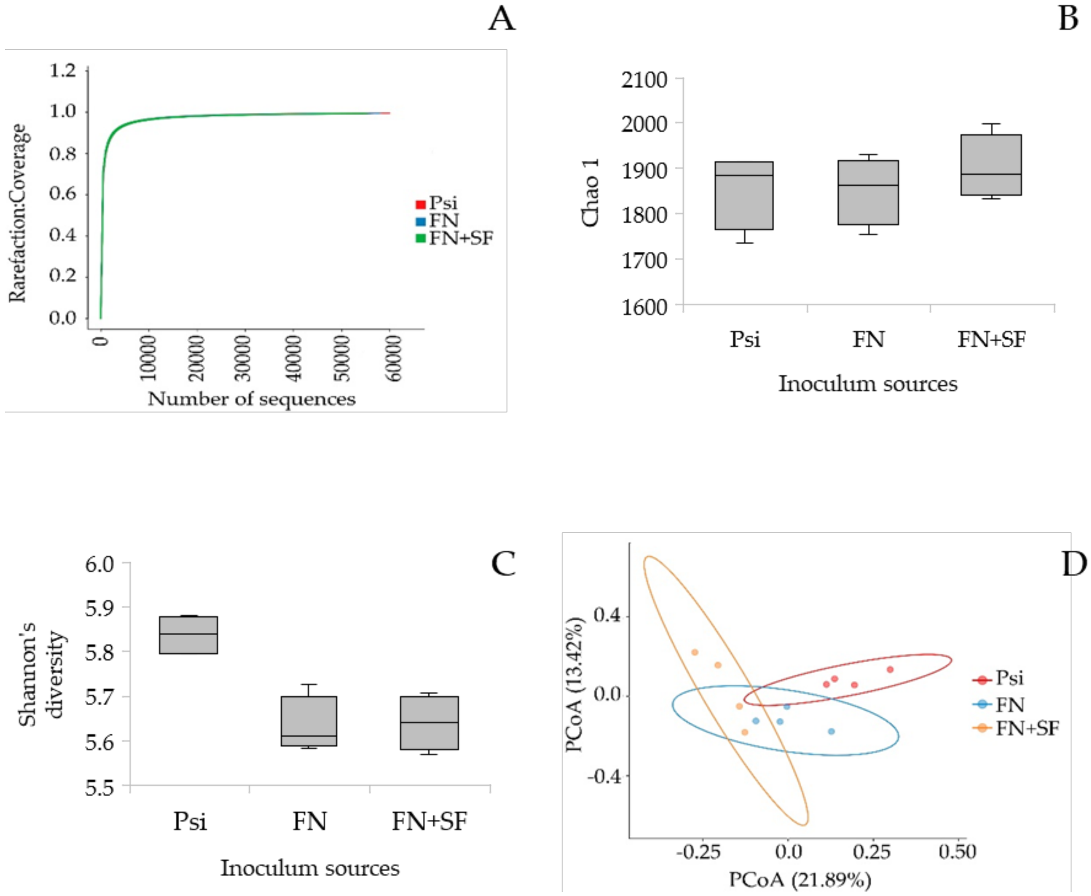

Figure 2. Diversity of bacterial communities associated with the rhizosphere of *Fraxinus uhdei* Wenz. Lingelsh. A: Rarefaction curves based on the observed diversity proportion; B: Chao 1 richness index; C: alpha diversity index; D: principal coordinate analysis (PCoA); circles enclosed by ellipses represent samples (biological replicates) from each treatment. Psi: Uninoculated plants; FN: plants inoculated with N_2-fixing *Azospirillum brasilense*; FN+SF: plants inoculated with N_2-fixing *A. brasilense* and phosphate-solubilizing *A. brasilense.*

community richness ranged from 1736 to 1998 taxa and was not influenced ($p < 0.05$) by inoculation with *A. brasilense* strains (Figure 2B). Alpha diversity was affected by inoculation treatments ($p \leq 0.05$), with greater diversity observed in the Psi rhizosphere compared to the FN and FN+SF treatments (Figure 2C), which showed the same levels of diversity (Shannon index).

Metagenomic analyses following inoculation with *A. brasilense* strains are limited and mostly focus on maize and rice crops. Reported results are contrasting, with effects ranging from null or minimal to clearly positive on bacterial community structure (Nievas *et al*., 2023). Inoculation with PGPR is usually associated with a decrease in alpha diversity. For example, in the rhizosphere of maize inoculated with commercial *A. brasilense* strains, a reduction in alpha diversity has been observed, even accompanied by modifications in the native microbiome. However, it has also been reported that greater diversity does not necessarily translate into a more favorable bacterial community for plant-PGPR interaction (Ferrarezi *et al*., 2023).

Beta diversity was also influenced by inoculation treatments (ANOSIM R = 0.59, $p \leq 0.05$). The principal coordinates analysis (PCoA) explained 35.3 % of the variation and showed a separation of bacterial communities between inoculated and uninoculated plants (Figure 2D). However, the overlap of the uninoculated plant ellipse with the inoculation treatment ellipses (95 % confidence interval) shows that changes in bacterial diversity are smaller.

The evidence available from sequencing methods indicates that the inoculation of plants with *Azospirillum* does not significantly alter microbial diversity but does favor microbial functional groups, such as diazotrophs (Renoud *et al*., 2022). This was observed with significant increases in the abundance of the genus *Azospirillum* (Figure 1B), such that *A. brasilense* strains exhibit the ability to interact with some bacterial groups in the *F. uhdei* rhizosphere.

Potential metabolic functions of bacterial communities

Prediction of potential metabolic functions at level three, based on species-specific KEGG pathways, showed that inoculation with *A. brasilense* had a significant effect ($p \leq 0.05$) on the relative abundance of 11 % of these functions (Figure 3). In particular, the

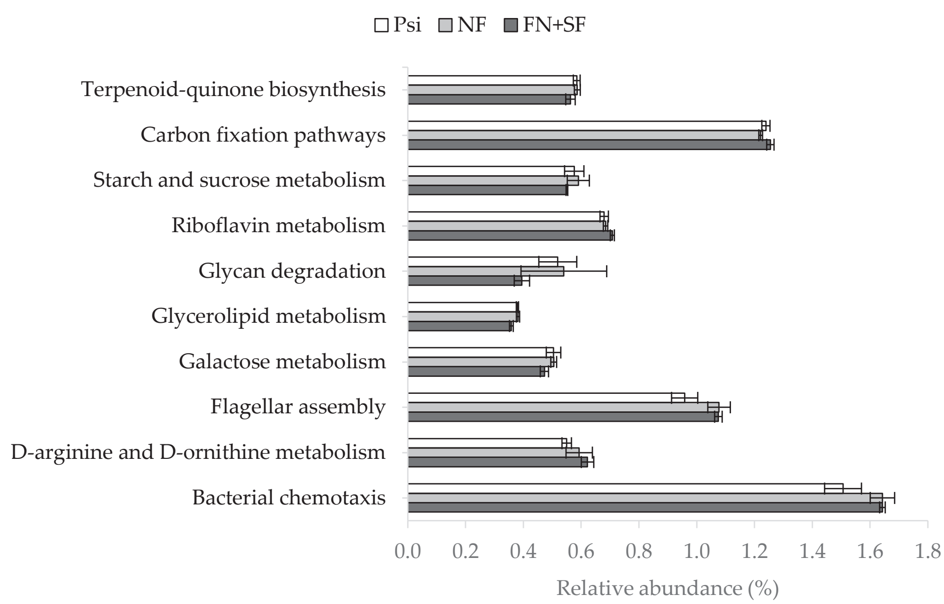

Figure 3. Relative abundance of metabolic functions influenced by inoculation treatments. Psi: Uninoculated plants; FN: plants inoculated with N_2-fixing *Azospirillum brasilense*; FN+SF: plants inoculated with N_2-fixing *A. brasilense* and phosphate-solubilizing *A. brasilense.*

chemotaxis function increased by 9 % with the FN treatment, while flagellum assembly increased by 12.4 % in FN+SF, both compared to Psi. The motility and chemotaxis of *A. brasilense* have been analyzed in real time during root colonization of various crops, showing that the accumulation of this PGPR in the rhizosphere responds to chemical gradients generated by root exudates that act as attractive or repellent signals (O'Neal *et al*., 2020). These results suggest that the strains used present a competitive chemotaxis that favors their colonization in the rhizosphere of *F. uhdei*.

The FN+SF treatment improved the abundance of functions related to disease resistance mechanisms and environmental adaptation. Riboflavin, D-arginine, and D-ornithine metabolism increased by 4.1 and 13.1 %, respectively. The remaining functions presented similar relative abundances between treatments. The metabolic functions of PGPR, particularly those focused on the production of secondary metabolites, are a strategy designed to occupy a niche close to the plant root so that they can access the supply of root exudates, establishing the PGPR-plant interaction (Naureen *et al*., 2022). In maize cultivation, the use of PGPR bacterium *Azospirillum* has favored the production of indoleacetic acid (IAA) in the rhizosphere, which has been associated with a higher abundance of certain metabolic functions (Coniglio *et al*., 2024). The *A. brasilense* FN strains used in this study are IAA producers (Carcaño-Montiel *et al*., 2006), which may be one of the factors that enhance the abundance of metabolic functions.

Seedling growth

The growth of *F. uhdei* seedlings showed a significant interaction ($p \leq 0.05$) between inoculation and controlled-release fertilizer, but only in stem biomass. The FN × 4 g L^{-1} substrate interaction generated greater accumulation of shoot biomass (9.8 ± 1.3 g), while the Psi × 2 g L^{-1} interaction recorded less accumulation (6 ± 0.3 g). The fertilizer dose affected ($p \leq 0.05$) the total height; the application of 4 g L^{-1} substrate showed greater height compared to the use of 2 g L^{-1} substrate (34.1 ± 1 and 31.2 ± 0.8 cm, respectively).

Regarding the inoculation treatment, an effect ($p \leq 0.05$) was found on stem diameter at the root collar, leaf area, root dry biomass, and total dry biomass (Table 1). The inoculant combination (FN+SF) generated greater growth, with increases of 11.4 % in stem diameter and 33.8 % in leaf area, compared to Psi. It also increased root biomass by 36.9 % and total biomass by 24.7 %, compared to the FN and SF treatments, respectively (Table 1). These values are lower than those reported for *Cecropia pachystachya* Trécul, where inoculation with *A. brasilense* increased leaf area and total biomass by 70.4 % (Calzavara *et al*., 2021). The differences are attributed to the higher growth rate of *C. pachystachya*, which is a warm-zone species with a high photosynthetic rate.

In the olive cultivar 'Arbequina,' under a pH gradient in the substrate, inoculation with *A. brasilense* strains almost doubled the total height of the plants compared to the controls, although it had no effect on the stem diameter (Boeni *et al*., 2024). This result contrasts with what was observed in *F. uhdei* plants, where inoculation did not favor height growth but did increase the stem diameter. The strains used in *F. uhdei*

Table 1. Growth in stem diameter at the root collar, leaf area, and dry biomass in *Fraxinus uhdei* Wenz. Lingelsh. seedlings inoculated with *Azospirillum brasilense* strains.

Sources of inoculum	Total height (cm)	Diameter (mm)	Leaf area (cm^2)	Shoot biomass (g)	Root biomass (g)	Total biomass (g)
Psi	31.3 ns	7.0 b	479.43 b	6.2 ns	5.2 ab	11.4 ab
FN	35.4 ns	7.3 ab	629.33 ab	8.1 ns	4.6 b	12.7 ab
SF	33.0 ns	7.5 ab	491.73 ab	6.6 ns	4.7 b	11.3 b
FN+SF	31.0 ns	7.8 a	641.60 a	7.8 ns	6.3 a	14.1 a
SEM	0.65	0.09	24.93	0.33	0.23	0.38

Mean values per column with a different letter are statistically different (p £ 0.05). Psi: Uninoculated plants; FN: plants inoculated with N_2-fixing *Azospirillum brasilense*; SF: plants inoculated with phosphate-solubilizing *A. brasilense*; FN+SF: plants inoculated with N_2-fixing *A. brasilense* and phosphate-solubilizing *A. brasilense*; SEM: standard error of the mean.

are producers of IAA, an auxin that in *F. uhdei* promotes root development whose absorption through the root system, followed by its translocation to the stem via xylem, stimulates cell elongation in the shoots (Kargapolova *et al*., 2020). Since the FN+SF strain combination significantly increased root biomass, it is likely that IAA accumulated in the root system and not in the shoot.

Seedling nutrition

The macronutrient content, with the exception of K, responded significantly ($p \leq 0.05$) to the interaction between inoculation and fertilizer dose. Compared to the Psi treatment, the FN × 4 g L^{-1} substrate interaction promoted greater nutrient accumulation in the whole plant, with increases of 24.4 % in N (Figure 4A), 44.7 % in P (Figure 4B), 29.6 % in Ca (Figure 4C), and 45.8 % in Mg (Figure 4D). Furthermore, the interaction revealed that when inocula were combined with the lowest fertilizer dose (treatment FN+SF × 2 g L^{-1}), the substrate tended to favor larger nutrient accumulation, showing significant differences ($p \leq 0.05$) in N, Ca, and Mg.

The results show a relationship with those found in *Araucaria angustifolia* (Bertol.) Kuntze, where inoculation with *A. brasilense* favored N and P contents (10.4 and 2.3 g kg^{-1}, respectively) compared to inoculation with *Bacillus subtilis* (8.1 and 1.7 g kg^{-1}, respectively) (Kondo *et al*., 2024). The benefits of PGPR in nutrient acquisition have been described in detail, showing their participation in the activation of ion transport systems within roots. In maize crops, *A. brasilense* strains excrete different amounts of NH_4^+ depending on the N source supplied (Pedrosa *et al*., 2019). The strains used in *F. uhdei* showed the ability to promote nutrient transfer, considering that the main source of mineral supply came from a controlled-release fertilizer. However, plants inoculated with FN had lower growth than those inoculated with FN+SF, which may be a factor in the higher nutrient content.

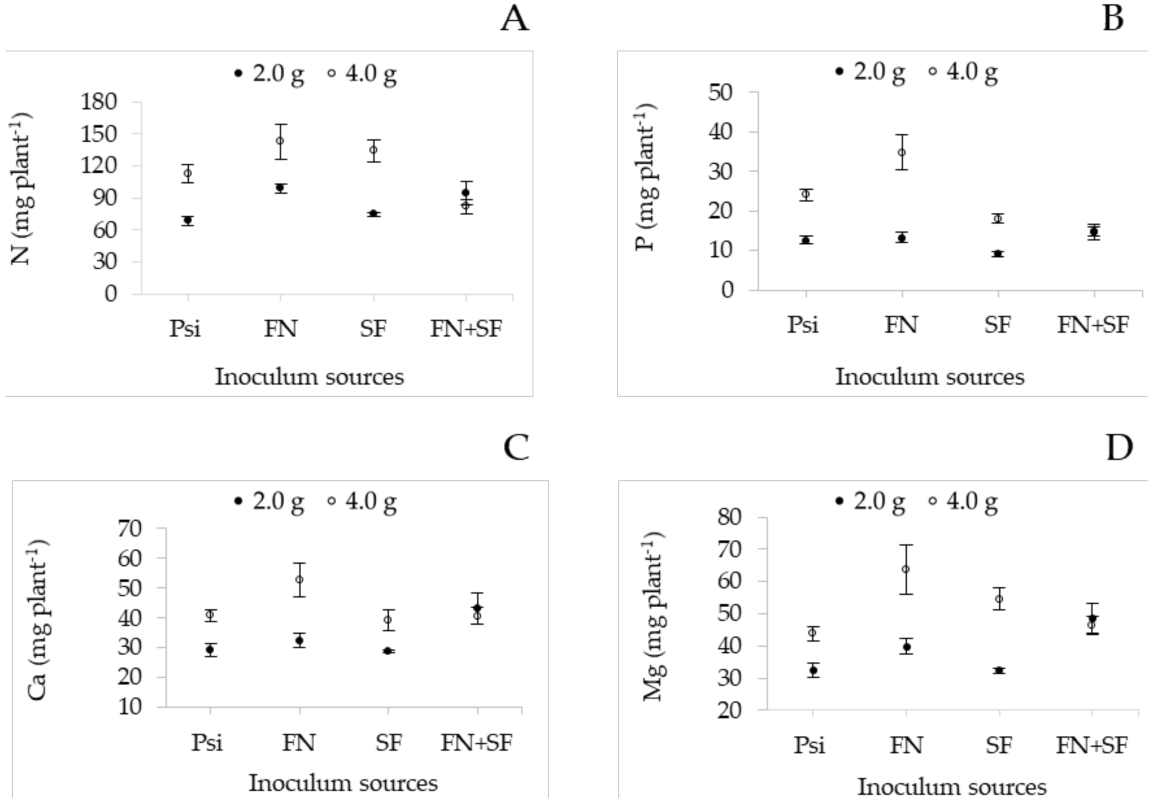

Figure 4. Macronutrient content in *Fraxinus uhdei* seedlings under the interaction of *Azospirillum brasilense* inocula × fertilizer dose (2 and 4 g L^{-1} of substrate). A: nitrogen content; B: phosphorus content; C: calcium content; D: magnesium content. Psi: Uninoculated plants; FN: plants inoculated with N_2-fixing *Azospirillum brasilense*; SF: plants inoculated with phosphate-solubilizing *A. brasilense*; FN+SF: plants inoculated with N_2-fixing *A. brasilense* and phosphate-solubilizing *A. brasilense*. The bars above the points indicate the standard error of the mean.

CONCLUSIONS

Inoculation with *Azospirillum brasilense* strains did not induce significant alteration in the overall composition of the rhizospheric bacterial community of *Fraxinus uhdei*. However, it influenced community diversity and the relative abundance of genes associated with potential metabolic functions. Seedlings inoculated with the combination of nitrogen-fixing and phosphate-solubilizing strains exhibited enhanced growth, whereas applications of the nitrogen-fixing strain alone resulted in higher macronutrient contents. These results indicated that the use of commercial *A. brasilense* strains as plant growth-promoting rhizobacteria may serve as an effective biotechnological strategy for the production of *F. uhdei* plants destined for urban environments. Furthermore, this approach is compatible with conventional inputs, such as peat-based substrates and controlled-release fertilizers, fostering the establishment of bacterial communities implicated in nutrient cycling.

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Agrociencia

PERCEPTION OF THE USE OF NEONICOTINOIDS, SPINOSAD, AND NEREISTOXIN IN INSECTS POLLINATING PAPAYA (*Carica papaya* L.), PERSIAN LEMON (*Citrus × latifolia* Tanaka), AND SOURSOP (*Anona muricata* L.) IN VERACRUZ, MEXICO

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ABSTRACT

Pollinating insects in tropical fruit trees are of utmost importance for flower pollination and ensuring fruit production. However, given the current climatic conditions and the chemical management practices, they are affected by the residual effects of neonicotinoids, spinosad, and nereistoxin on the flowering of papaya (*Carica papaya* L.), Persian lemon (*Citrus × latifolia* Tanaka), and soursop (*Annona muricata* L.). The overuse of these insecticides has led to the decline of pollinating insects such as honeybees. The objective of this study was to assess the use of neonicotinoid insecticides spinosad and nereistoxin during flowering of papaya, Persian lemon, and soursop, and evaluate stakeholders' perceptions of the impact of pollinator population density in central Veracruz, Mexico. A survey was carried out among papaya, Persian lemon, and soursop producers through a questionnaire with open and closed questions. The data were analyzed using frequency and multivariate analyses with the statistical package STATISTICA version 7.0. Results show that producers use imidacloprid, thiamethoxam, acetamiprid, spinosad, and the nereistoxin known as thiocyclam to control mites and insect pests. Producers observed damage to bees and beneficial insects after applying these chemicals. In conclusion, the management and application of toxic insecticides for pest control pose a significant risk to pollinating insects.

Keywords: Tropical fruit trees, insecticides, toxicity, residues, *Apis mellifera* L.

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INTRODUCTION

Due to climate change, natural pollinator populations are declining, which has implications for ecosystems and agroecosystems. Mutualistic interactions, such as plant-pollinator associations, are critical for pollination. Plants benefit from the visit of organisms that act as pollinators by carrying pollen to other flowers (Maglianesi-Sandoz, 2016). According to estimates, 70 % of tropical crops produce significantly more when pollinators visit their flowers (Garibaldi *et al*., 2012).

The decline of honey bee (*Apis mellifera* L.) colonies is attributed to multiple factors, such as the use of pesticides, genetically modified crops, habitat loss, and the presence of diseases or parasites. Among the pesticides, neonicotinoids stand out for their toxicity, as they are systemic neurotoxic insecticides that accumulate in pollen, nectar, and guttation fluids of plants, increasing their impact on pollinators (Fairbrother *et al*., 2014). Their translocation to floral tissues causes residual effects that generate both lethal and sublethal mortality in different bee species.

Riano-Jiménez and Cure (2016) evaluated the toxicity of the insecticides imidacloprid, spinosad, and thiocyclam hydrogen oxalate (nereistoxin) in neotropical bees of the species *Bombus atratus*, determining their median lethal doses (LD50). Imidacloprid was the most toxic, with a topical and oral LD50 of 0.000048 and 0.00001 mg L^{-1}, equivalent to 0.048 and 0.01 mg per bee, respectively. For thiocyclam hydrogen oxalate, the topical and oral LD50 were 0.000244 and 0.000056 mg L^{-1} (0.244 and 0.056 μg per bee). In the case of spinosad, the oral LD50 reached 0.0002 mg L^{-1} (0.2 μg per bee).

Neonicotinoids present in pollen and nectar can cause mortality in crop-pollinating insects (Fairbrother *et al*., 2014). Nereistoxin, a neurotoxic substance isolated from the marine annelid *Lumbriconereis heteropoda*, acts as a poison for weevils and caterpillars and may pose a risk to pollinators and humans (Kumar *et al*., 2011). Since the mid-20th century, honeybee populations maintained in artificial hives have declined. In contrast, managed colonies in Asia, Africa, and Australia have increased since 1991 (Fairbrother *et al*., 2014). In Mexico, studies on papaya (*Carica papaya* L.) from Cotaxtla, Veracruz, showed residues of thiamethoxam in water, plant, and fruit matrices, which poses an additional risk to pollinators associated with flowering (Megchún-García *et al*., 2019, 2024).

Chemical insecticides affect pollinators through different mechanisms of action, either through direct toxicity that causes mortality or sublethal effects that alter their behavior, reproduction, navigational ability, and immune function (Maggi and Chreil, 2023). During flowering in tropical fruit trees, the movement of pollinators favors the transfer of male gametes and ensures the sexual reproduction of plants. If pollinators are scarce or inefficient, pollination can fail due to poor quality and quantity of the pollen transported, as well as inadequate depositing on the stigma that prevents germination (Garibaldi *et al*., 2012).

In Mexico, papaya and Persian lemon (*Citrus* × *latifolia* Tanaka) are crops of national and international economic importance, while emerging fruit trees such as soursop (*Annona muricata* L.) have significant market potential (Megchún-García *et al*., 2023). In papaya, physiological triggers regulate primordia formation and flower opening in the androecious, andromonoecious, and gynoecious sexual forms. Stigma receptivity begins on the same day as flower opening, at which time flowers can be visited by pest insects or beneficial pollinator-associated insects, such as stingless bees (Pares *et al*., 2002; Aspeitia-Echegaray *et al*., 2014; Hoyos and Hurtado-Salazar, 2017; Megchún-García *et al*., 2018).

In the case of lemon, there are numerous floral flows that are continuously visited by pollinators throughout the year (Almaguer-Vargas *et al*., 2011). Lemon tree pollination is carried out by entomophilous insects, primarily bees and flies. Citrus fruits vary greatly in their dependence on pollinators, with cases such as tangerines that are completely dependent on them (Moreno *et al*., 2024).

In soursop, natural pollination is limited by dichogamy or protogyny, resulting in poor and erratic fruit production. During this stage, pollinating insects such as beetles and ants play a key role during anthesis (Rodríguez *et al*., 2010), reducing dependence on manual pollination to ensure fruit production. However, the use of insecticides such as neonicotinoids, spinosad, and nereistoxin during flowering has led to a decline in pollinator populations. Therefore, the objective of this study was to assess the use of these insecticides during flowering of these fruit trees and analyze local stakeholders' perceptions of their impact on pollinator population density in central Veracruz, Mexico.

MATERIALS AND METHODS

The study was carried out in the central region of the state of Veracruz, Mexico, in the municipalities of Cotaxtla, Tlalixcoyan, and Tlapacoyan (Figure 1). A technical survey was conducted in the towns of Avión, Colonia Ejidal, Mata Tambor, Los Bajos de Tlachiconal, Los Bajos Platanal, Cotaxtla, ejido Anexo Mundo Nuevo, and La Palmilla to identify the study crops. The municipality of Cotaxtla produces 1643.5 ha of lemon, 737.5 ha of papaya, and 7 ha of soursop; the municipality of Tlalixcoyan has 543.71 ha of papaya and 423 ha of lemon; and the municipality of Tlapacoyan produces 3758 ha of lemon and 8 ha of soursop (SIAP, 2024).

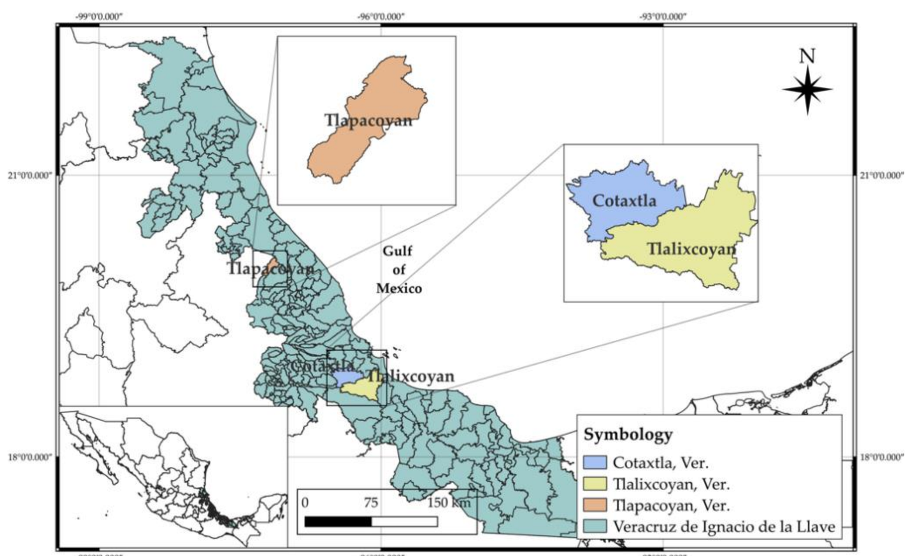

Figure 1. Location of the study area in municipalities of Veracruz, Mexico.

A survey was conducted to gather information on the technical management of neonicotinoid insecticides, spinosad, and nereistoxin, considered the main compounds that affect natural pollinators, particularly bees. Non-probability sampling was used, concentrating on the identification of key informants, including prominent producers and agricultural stakeholders. The sample interviewed consisted of 66 producers dedicated to the cultivation of papaya, Persian lemon, and soursop, with crop areas ranging from 1 to 10 ha. A semi-structured questionnaire with open and closed questions was used, and an infographic was created to classify the insecticides based on the most common brand names for these fruit trees.

The questionnaire was validated using SPSS version 25 software (IBM SPSS Statistics), with the collaboration of five judges who assessed the clarity and comprehension of the questions on a scale of 1 to 5, considering their correct wording. A pilot test was also conducted to rule out poorly reasoned questions. A technical survey was carried out in the field, starting in the municipalities of Cotaxtla, Tlalixcoyan, and Tlapacoyan. The activity began in April and ended in September 2024.

The study variables evaluated were: production system; type of fruit tree (papaya, Persian lemon, soursop); cultivated area (ha); distance between plants and rows (m); planting date; flowering and harvest periods; presence of pests; management and application of insecticides, including the type of insecticide that affects natural pollinators, time of application, product mix, frequency and effectiveness; insecticides that damage crops or cause mortality in pollinating bees; phenology of fruit trees and application of insecticides according to phenological stage; application and protection equipment; unused insecticides and reasons for not using them; and flower drop on fruit trees, as well as problems present in the ecosystem. The data were coded in Microsoft Excel 2009 and analyzed in Statistica version 7. Frequency and multivariate principal component analyses were carried out.

RESULTS AND DISCUSSION

According to the survey, 84.8 % of producers are dedicated to growing Persian lemons. Of the total producers, 69.6 % operate an intensive monoculture production system, while the rest establish crops in association with grasses and vegetables, such as corn, watermelon, coconut, and banana (Table 1). The growing demand and consumption

Table 1. Producer distribution by crop and production system in the central area of Veracruz, Mexico.

Tropical fruit tree	Frequency	Producers (%)	Production system	Frequency	Producers (%)
Papaya (*Carica papaya* L.)	9	13.6	Associated cropping	19	28.8
Persian Lemon (*Citrus* × *latifolia* Tanaka)	56	84.8	Monoculture	46	69.7
Soursop (*Annona muricata* L.)	1	1.6	In rotation	1	1.5
Total	66	100	Total	66	100

of tropical fruits like lemons is increasing worldwide, particularly in Europe, at an average rate of 6 % per year. Pollinating insects are essential for fruit production as they ensure pollination rates and fruit set (de la Peña-Alonso *et al*., 2018). However, the main cause of pollinator decline is habitat loss, as more than 40 % of the ice-free land surface has been modified by humans for agricultural purposes. This alteration limits the availability of food and nesting sites, hindering pollinator adaptation (Bartomeus and Bosch, 2018).

It was observed that producers use a specific group of chemical insecticides as part of integrated pest management in lemon, papaya, and soursop crops. Among the insecticides that affect pollinators, 3 % of producers use imidacloprid and abamectin, while 2 % use only imidacloprid. Most producers use neonicotinoids such as acetamiprid, imidacloprid, and thiamethoxam.

Imidacloprid is primarily used to control leaf miner (*Phyllocnistis citrella*), aphids, mites, and red spider mites (*Tetranychus urticae*). In the case of spinosad, it is often combined with other active ingredients, such as imidacloprid, dimethoate, and cypermethrin. Spinetoram, a fermentation product derived from the actinobacterium *Saccharopolyspora spinosa*, was also used. Its active component, spinosyn, exhibits effects similar to those of spinosad. Overall, 48.9 % of producers use imidacloprid for agronomic pest management (Table 2).

The chemical structure of neonicotinoids gives them systemic properties, allowing for complete plant protection by distribution throughout the vascular system, reaching the pollen and nectar. This characteristic can lead to the presence of residues of imidacloprid, clothianidin, and thiamethoxam in wildflowers, which pose a risk to pollinators such as domestic, commercial, and wild bees, hoverflies, wasps, beetles, and ants present in tropical fruit trees (Botías and Sánchez-Bayo, 2018). Pollinators play an important role in floral evolution, particularly in angiosperms, by promoting mutualistic interactions in pollination processes. However, external factors such as exposure to neonicotinoids negatively affect these processes (Simón-Porcar *et al*., 2018; Megchún-García *et al*., 2019).

In studies on honeybees (*Apis cerana* Fabricius), exposure to more than two pesticide combinations (imidacloprid, chlorobenzamide, and glyphosate) reduced bee flight time and distance. Furthermore, transcriptomic results indicated that concentrations of 0.001 mg L^{-1} of imidacloprid could affect the survival rate of queen bees (Guo *et al*., 2025). Other studies indicated that the neonicotinoid acetamiprid significantly altered the gut microbial community in adult and larval bees, suggesting that it directly affects bee health (Su *et al*., 2024).

In the interview, 56 % of producers reported not perceiving any harm to pollinating insects due to insecticides (Figure 2). Of those who apply neonicotinoid insecticides, 16.6 % identified imidacloprid as the main cause of harm to natural pollinators. Meanwhile, 9 % associated spinosad with the damage. In this case, 1.5 % of producers reported using nereistoxin (thiocyclam), usually in a mixture with other active ingredients to control whiteflies (*Bemisia tabaci*), red spider mites (*Tetranychus urticarie*), *Diaphorina citri*, thrips (Thysanoptera), and ants (Formicidae).

Table 2. Insecticides used by producers for pest control in papaya (*Carica papaya* L.), Persian lemon (*Citrus* × *latifolia* Tanaka), and soursop (*Annona muricata* L.) and their potential impact on pollinators.

Groups of insecticides used	Frequency (number of interviewees)	Fruit tree producers (%)
Cypermethrin, chlorpyrifos ethyl	5	7.6
No insecticides used	5	7.6
Cypermethrin	3	4.5
Imidacloprid, abamectin	3	4.5
Abamectin, chlorpyrifos ethyl	2	3
Abamectin, imidacloprid, spinetoram	2	3
Abamectin, imidacloprid, tolfenpyrad	2	3
Acetamiprid, spinetoram etaxazole	2	3
Imidacloprid	2	3
Abamectin	1	1.5
Abamectin, chlorpyrifos ethyl, carbofuran	1	1.5
Abamectin, chlorpyrifos ethyl, diazinon	1	1.5
Abamectin, dimethoate, diazinon	1	1.5
Abamectin, pinol, soap	1	1.5
Abamectin, spinetoram, spirotetramat	1	1.5
Bifenazate, organic insecticide	1	1.5
Cypermethrin, pyraclostrobin	1	1.5
Chlorpyrifos ethyl, carbaryl, cypermethrin	1	1.5
Diazinon, malathion, dimethoate	1	1.5
Botanical extracts	1	1.5
Imidacloprid, abamectin, elemental sulfur	1	1.5
Imidacloprid, abamectin, bifenazate	1	1.5
Imidacloprid, abamectin, cypermethrin	1	1.5
Imidacloprid, abamectin, chlorpyrifos ethyl	1	1.5
Imidacloprid, abamectin, diazinon	1	1.5
Imidacloprid, abamectin, diazinon, chlorpyrifos ethyl, spinetoram	1	1.5
Imidacloprid, abamectin, dimethoate	1	1.5
Imidacloprid, abamectin, soap	1	1.5
Imidacloprid, abamectin, malathion	1	1.5
Imidacloprid, abamectin, sulfoxaflor, chlorpyrifos ethyl	1	1.5
Imidacloprid, elemental sulfur, malathion	1	1.5
Imidacloprid, cypermethrin	1	1.5
Imidacloprid, chlorpyrifos ethyl, paraffinic oil	1	1.5
Imidacloprid, chlorpyrifos ethyl, diazinon	1	1.5
Imidacloprid, chlorpyrifos ethyl, organic insecticide	1	1.5
Imidacloprid, dimethoate, cypermethrin	1	1.5
Imidacloprid, neem extract	1	1.5
Imidacloprid, organic insecticide	1	1.5
Imidacloprid, malathion, chlorpyrifos ethyl	1	1.5
Imidacloprid, spinetoram, carbofuran	1	1.5
Imidacloprid, spinosad	1	1.5
Imidacloprid, spinosad, abamectin	1	1.5
Organic insecticide	1	1.5
Methamidophos, spinetoram, cypermethrin, abamectin, chlorpyrifos ethyl	1	1.5
Spinetoram	1	1.5
Spinosad	1	1.5
Spinosad, cypermethrin, permethrin	1	1.5
Spinosad, dimethoate	1	1.5
Thiamethoxam, organic insecticide	1	1.5
Total	66	100

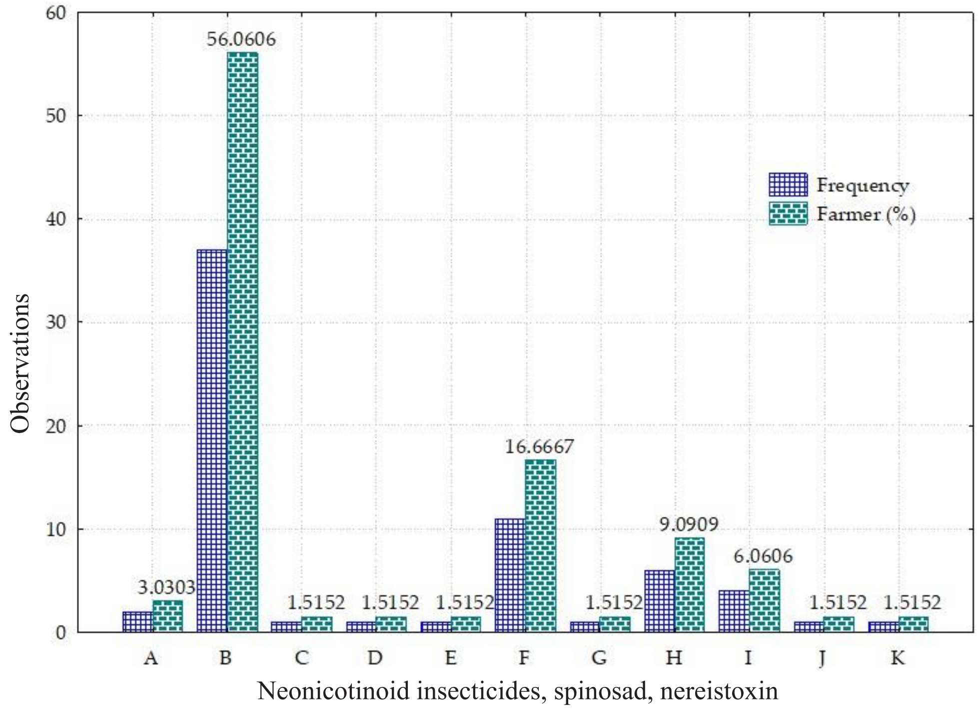

Figure 2. Frequency and percentage of producers who identified neonicotinoid insecticides, spinosad, and nereistoxin as causing mortality in natural pollinators in tropical fruit crops. A: dinotefuran; B: no perception of effects; C: thiamethoxam, lambda-cyhalothrin; D: thiamethoxam, thiocyclam; E: thiamethoxam; F: imidacloprid; G: clothianidin, thiamethoxam, thiocyclam, tolfenpyrad; H: spinosad; I: tolfenpyrad; J: diazinon; K: imidacloprid, tolfenpyrad.

Pollinators can be exposed to insecticides through direct contact with airborne particles or treated plant surfaces, through the ingestion of pollen, nectar, and contaminated water, or through inhalation of volatile pesticides (Botías and Sánchez-Bayo, 2018). One of the risks of applying neonicotinoids such as imidacloprid is their negative effect on natural enemies, such as the predator *Arma chinensis*. This shows that although imidacloprid is effective in pest control, it can also harm beneficial insects (Cheng *et al*., 2025).

Of the insecticides that kill bees during the flowering stage of tropical fruit trees, producers mentioned spinosad and clothianidin in order of importance with 3 %, followed by imidacloprid and other active ingredients with 1.5 % (Figure 3). Spinosad has demonstrated low toxicity to bees when allowed to dry on plant foliage for 3 h (Mayes *et al*., 2003). Bumblebees have been reported to be more vulnerable than honeybees to the presence of imidacloprid in pollen and nectar (Martin-Culma and Arenas-Suárez, 2018). The phenomenon of bee poisoning, known as Colony Collapse Disorder, is caused by exposure to neonicotinoids and pyrethroids such as cypermethrin, which have long-term impacts on honeybee populations and

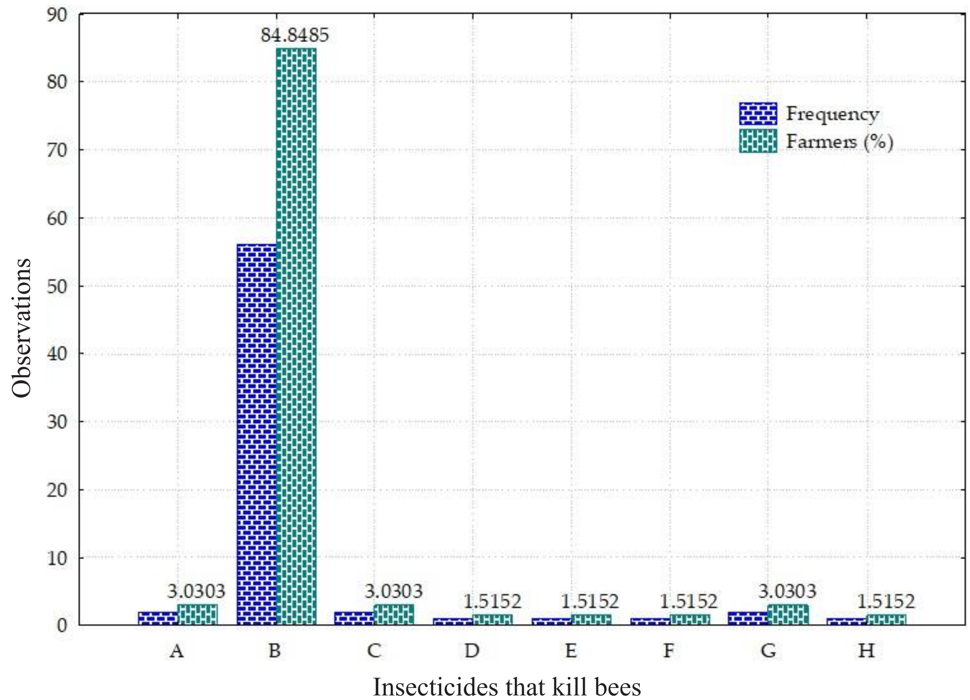

Figure 3. Frequency and percentage of producers who identified insecticides causing mortality in honeybees (*Apis mellifera* L.) during flowering of tropical fruit trees. A: spinosad, clothianidin; B: no effects perceived; C: cypermethrin; D: lambda-cyhalothrin; E: imidacloprid, abamectin, spinetoram; F: abamectin; G: all insecticides; H: tolfenpyrad.

communities and wild pollinators. Therefore, in countries like Italy, permethrin and cypermethrin are banned in agriculture (Gasparini *et al*., 2025).

From the total of producers, 75.7 % reported no damage from the application of chemically synthesized insecticides, but 4.5 % argued that bees are killed when insecticides are applied at different phenological stages. Around 1.5 % identified the death of natural predators, and 3 % observed the death of beneficial insects and fish. Similarly, 4.5 % of producers reported flower drop in all three crops (Figure 4).

Pollinator research indicates the existence of secondary pollinators, or anthophilous insects, including beetles, lepidopterans (butterflies), flies, wasps, and ants. Although they are less efficient pollinators, their flower visitation intensity contributes in a complementary way to bees (Stefanescu *et al*., 2018). Factors such as land-use change, habitat loss, pollution, and climate change have accelerated the decline of pollinators. In tropical regions, the most important pollinating insects include bees, wasps, and ants (Hymenoptera), flies (Diptera), butterflies and moths (Lepidoptera), and beetles (Coleoptera). In addition, there are vertebrate pollinators such as nectarivorous birds (Trochilidae), nectarines (Nectariniidae), honey eaters (Meliphagidae), and parrots (Psittacidae), as well as bats that pollinate agaves in deserts and tropical rainforests (Meléndez-Ramírez *et al*., 2020).

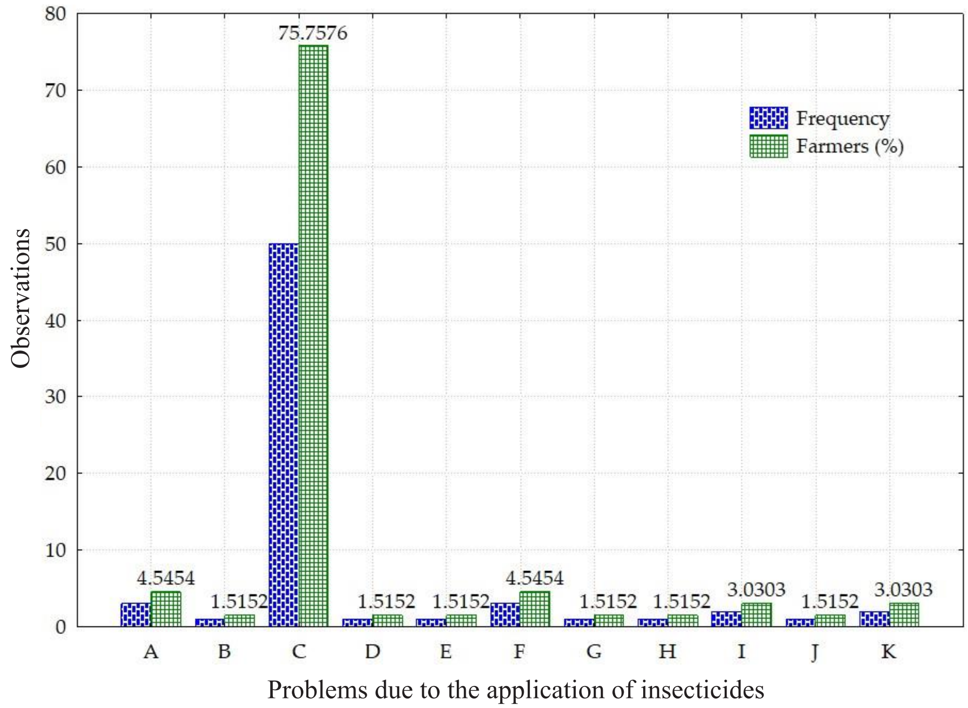

Figure 4. Frequency and percentage of observations reported by producers regarding problems caused by the application of synthetic chemical insecticides on tropical fruit trees. A: flower drop; B: plant burning; C: no damage perceived; D: flower drop and death of butterflies; E: death of bees and butterflies; F: death of bees; G: flower and fruit drop; H: death of natural predators; I: insecticide resistance; J: attraction of more insects; K: death of beneficial insects and fish.

In the multivariate principal components analysis, a positive relationship was observed between insecticide application equipment and method according to the phenological stage (Figure 5), which directly influences insecticide effectiveness within integrated pest management. On the other hand, a negative relationship was observed with insecticide management, pest presence, active ingredient mix, and planting date. These results suggest that the level of damage caused by systemic insecticides such as neonicotinoids depends on the method and management of application in the field.

Previous studies have shown that the inappropriate use of neonicotinoids affects bees and other beneficial insects, reducing their survival and causing mortality due to contact or ingestion, mainly through consumption of contaminated nectar, pollen, resins, or water (Cajamarca *et al*., 2020). This remarks the need to implement local bee poisoning surveillance and prevention programs (Gasparini *et al*., 2025).

Study variables	
Production systems:	SP
Fruit trees (Papaya, Persian lemon, Soursop):	FRPLG
Area (ha):	SUP
Distance between plants (m):	DP
Distance between rows (m):	DH
Sowing date:	FS
Flowering in the year:	FA
Harvest (year):	COS
Pests in the crop:	PLC
Insecticide management:	MI
Insecticide that harms natural pollinators:	IDPN
Time of application of insecticides:	HRAI
Phenology of tropical fruit trees:	FFT
Application of insecticides by phenological stage:	AIEF
Insecticide mixture:	MEI
Application frequency:	FA
Most effective insecticides:	IE
Insecticides that cause damage to crops:	ICDC
Type of problems present in the ecosystem:	TPPE
Insecticides that kill pollinating bees:	ICMAP
Application equipment:	EA
Protective equipment:	EP
Insecticides that do not use:	INU
Flower drop on fruit trees:	CFF
Reason for not using it:	MNU

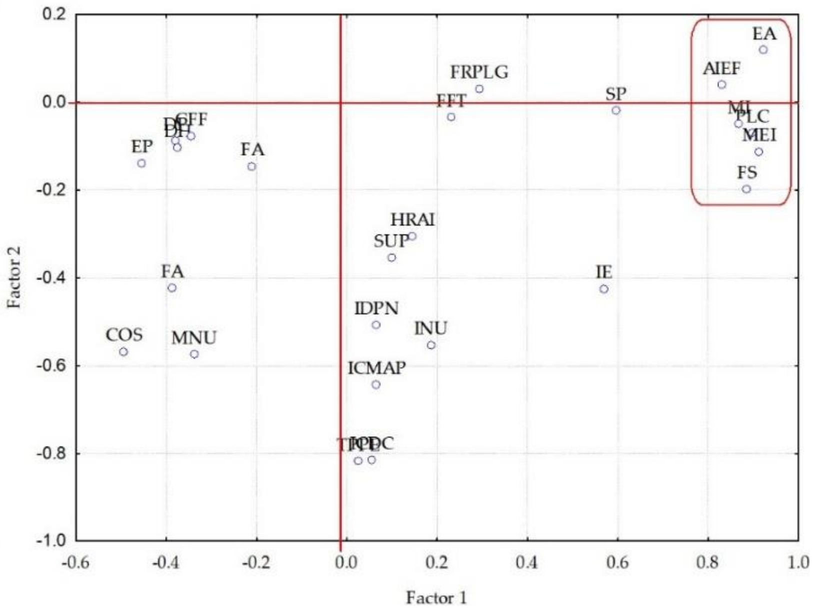

Figure 5. Multivariate principal components analysis by two factors for the management of neonicotinoid insecticides, spinosad, and nereistoxin in papaya (*Carica papaya* L.), Persian lemon (*Citrus* × *latifolia* Tanaka), and soursop (*Annona muricata* L.) cultivation.

CONCLUSIONS

Producers use insecticides that affect pollinating insect populations, such as neonicotinoids and spinosad, although few realize the impact these chemical compounds have on beneficial insects associated with flowering and the ecosystem. Imidacloprid is the most widely used, generally in combination with other active ingredients, due to its high effectiveness in controlling pests in the crops analyzed. Other emerging insecticides, such as nereistoxin, are beginning to gain relevance in integrated pest management, making it important to conduct residual studies on flowers to assess their impact on natural pollinators. Therefore, there is a potential risk of frequent damage to pollinating insects if management and use of toxic insecticides in fruit crops are not properly regulated.

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Agrociencia

LANDSCAPE PERCEPTION OF A NATURALISTIC PLANTING IN AN ECOTOURISM SPACE

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ABSTRACT

Naturalistic gardens have recently sparked growing interest due to the social and environmental benefits they offer. This type of garden is characterized by the use of native plants, an organic and fluid design, and layouts that avoid straight lines that recreate patterns found in nature, such as flower meadows. In contrast, conventional gardens prioritize aesthetic criteria without considering the required inputs or their environmental impact. The aim of this study was to evaluate visitors' perceptions of a naturalistic planting with herbaceous species in an ecotourism space in order to propose more sustainable and socially relevant alternatives, while contributing to the discussion on the social acceptance and potential of naturalistic landscaping in Mexico. The study was conducted at the Playa La Junta tourism development in the municipality of Amatlán de los Reyes, Veracruz, to assess the feasibility of its implementation as an ornamental resource that contributes to the area's tourist appeal. In January 2018, a planting was established, consisting of five ornamental herbaceous plant species: *Lantana camara* L., *Salvia coccinea* Buc'hoz ex Etl., *Salvia leucantha* Cav., *Ruellia simplex* C. Wright, and *Zinnia elegans* Jacq. In March 2018, during the flowering period, a survey was given to 150 on-site visitors to assess their perceptions. The data were analyzed using descriptive statistics and analysis of variance. The results revealed statistically significant differences ($p \leq 0.05$) in perception levels of the naturalistic planting. Favorable perceptions were found among females and people between the ages of 30 and 60. The most highly valued aspects were the variety of colors and species diversity. The flower meadow obtained a high rating in terms of perception, which highlights its ornamental potential for nature tourism ventures and its capacity to attract more visitors.

Keywords: garden, ornamental potential, landscape design, landscaping.

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INTRODUCTION

The perception of green areas is key to understanding the relevance of naturalistic gardens. This perception is based on people's senses and feelings, influenced by their natural and cultural identity. This connection generates a sense of ownership and belonging, promoting emotional and physical balance (García-Serrano *et al*., 2023). Previous studies have shown that green areas with trees and shrubs improve the visual quality of the environment, which is directly associated with psychological and emotional well-being, while also promoting the restoration of physical health of those who observe or interact with them (Zhang *et al*., 2024). Interaction with and contemplation of natural green areas promotes a more active and healthier lifestyle, positively impacting quality of life (Guarda-Saavedra *et al*., 2022). This physical and psychological well-being is a fundamental criterion to consider in landscape design.

While landscape design often focuses on enhancing the aesthetics of a site, it is essential that it also fulfills relevant ecosystem and cultural functions (Nassauer, 1993). From this perspective, naturalistic-oriented design incorporates wild vegetation, especially herbaceous plants of varied colors, in organic arrangements that alternate flowering periods, generate textural nuances, and apply ecological design principles. This approach seeks to enhance ornamental value while also contributing to people's quality of life (Southon *et al*., 2017; García-Albarado and Dunnett, 2009).

Mexico has a wide variety of plants, of which approximately 4220 have ornamental potential (Munguía-Lino *et al*., 2010). The state of Veracruz, one of the most floristically diverse in the country, is home to around 7855 species (Castillo-Campos *et al*., 2011), with herbaceous plants accounting for more than 50 % of them. This richness represents a wide range of possibilities for creating plant palettes with a variety of colors and textures for parks and gardens.

Naturalistic planting design has become a growing trend among landscape professionals, considered both an ethical and aesthetic advancements (Özgüner and Kendle, 2006). It is a dynamic style that promotes plant succession and phenological changes throughout the seasons and can generally be established at lower costs than formal systems (Dunnett and Clayden, 2007). Wild plants with ornamental potential have proven to be ideal for these alternative landscapes, as they require less management and agricultural inputs (García-Albarado *et al*., 2013). Furthermore, in a context of climate change and reduced resources allocated to the maintenance of green areas, naturalistic design has gained relevance by reducing costs and maximizing ecosystem benefits (Hitchmough, 2011).

Naturalistic plantings are not always perceived positively. Some sectors consider them less aesthetic, neglected, or dangerous, preferring formal designs with defined geometries (Özgüner and Kendle, 2006). However, other studies have revealed that municipal technicians and citizens show a preference for more naturalized public spaces, with spontaneous vegetation or meadows that convey a sense of wildness (de la Fuente-de Val, 2022). To improve social acceptance, the design should demonstrate its intentional nature through signs of care, such as trimmed edges, sculptural elements, or discreet signage (García-Albarado *et al*., 2021).

Landscape perception is a complex and multidisciplinary field of study, essential in planning for tourism purposes (Nogué i Font, 1992). Recent research confirms that people associate green spaces with benefits such as creating wildlife habitats, reducing stress, improving mood, and evoking pleasant memories (Nevárez-Favela *et al*., 2023). Therefore, this study contributes to the debate on naturalistic landscaping in Mexico by providing empirical evidence on the social perception of a planting with wild herbaceous species in an ecotourism context.

MATERIALS AND METHODS

Location of the study area

The study was conducted at the Playa La Junta ecotourism venture (18° 55′ 10.05″ N and 96° 52′ 31.91″ W, at an altitude of 740 m), considered one of the most representative nature tourism sites in the municipality of Amatlán de los Reyes, Veracruz (Gobierno del Estado de Veracruz, 2022). Fieldwork was carried out in March 2018, when the site registered a period of steady visitor influx that facilitated the implementation of the perception survey. Playa La Junta is located in the community of Ojo de Agua, 9.3 km from the municipal seat of Amatlán de los Reyes, within a rural setting that favors the development of ecotourism activities. The climate is classified as warm with summer rainfall, with an average annual temperature of 18 °C and mean precipitation of 1807.3 mm. The potential natural vegetation corresponds to a sub-deciduous tropical forest, currently fragmented due to agricultural and ecotourism activities.

Plant palette selection

The selection and propagation of the plant palette included five ornamental species. Although this number may be small, this decision followed a methodological simplification criterion aimed at facilitating experimental management, assessing initial visitor responses, and establishing a representative baseline of the local flora with ornamental and ecological value. Although this number is smaller than what is typically employed in naturalistic gardens, it provided a controlled framework for analyzing public perceptions of this design approach. The selection criteria included: i) preference for local species, ii) diversity in floral forms, iii) variety of colors, and iv) maximum height of approximately 1 m.

The selected species were *Lantana camara* L., *Salvia coccinea* Buc'hoz ex Etl., *Salvia leucantha* Cav., *Ruellia simplex* C. Wright, *and Zinnia elegans* Jacq. The environmental conditions of the site influence the selection of species and the planting layout. Although the species used are all native, questions arise regarding the potential effects of promoting assemblages that do not strictly reflect local natural associations. In this sense, the design corresponds more to the notion of an ornamental "pictorial meadow." Therefore, only a single layer ranging from 80 to 100 cm in height was used. Propagation was carried out using sexual (by seed) and asexual (by cuttings) methods according to each species (Table 1). This phase took place in the nursery at the

Table 1. Species used in the naturalistic planting established in Amatlán de los Reyes, Veracruz, Mexico.

Species	Family	Color	Production	Floral shape	Propagation method
Lantana camara L.	Verbenaceae	Yellow, red, pink	Nectar	Open	Asexual
Salvia coccinea Buc'hoz ex Etl.	Lamiaceae	White, red, pink	Nectar	Tubular	Asexual
Salvia leucantha Cav.	Lamiaceae	Lilac	Nectar	Tubular	Asexual
Ruellia simplex C. Wright	Acanthaceae	Lilac, pink	Nectar	Bell-shaped	Asexual
Zinnia elegans Jacq.	Asteraceae	Orange, red, pink	Pollen and Nectar	Open	Sexual

Postgraduate College Córdoba Campus, located in the municipality of Amatlán de los Reyes, Veracruz, starting in October 2017.

Planting design and establishment

The naturalistic planting was designed and established in January 2018, covering an area of 7.2 m^2. For the garden design, the plants were randomly distributed, with the tallest and largest species in the center and the smallest ones on the periphery (García-Albarado and Dunnett, 2009). The site was located in an open space near the parking area, allowing easy access and a preferential view from the entrance. The combination of textures, heights, and shades of the selected species created a dynamic and visually appealing composition (Figure 1).

Recommendations established by Nassauer (1993) were considered in the design of the naturalistic planting. Small, discreet signs were placed among the plants, instructing visitors not to cut the flowers, as they were intentionally designed for research purposes. The grass surrounding the planting was also mowed.

The selection of the plant palette was adequately justified in terms of chromatic and formal diversity, which made it possible to create a planting that was attractive to visitors. However, since it was not compared with other types of design (e.g., a conventional ornamental arrangement or an area of spontaneous vegetation), it is not possible to conclusively attribute the rating recorded to the naturalistic style itself. It is possible that part of the positive rating stems simply from the presence of flowering plants at their peak bloom period. Recognizing this limitation is important and suggests that future research should incorporate comparisons between different planting styles to differentiate the effect of the naturalistic approach itself.

Perception study

The perception of the planting was determined based on the participants' assessment of the established naturalistic planting in full bloom. Questionnaires were administered on-site (n = 150) as an evaluation tool. Participants were randomly selected using probability sampling and voluntarily agreed to participate during their visit to the site. The questionnaires were distributed between 10:00 a.m. and 4:00 p.m. on March

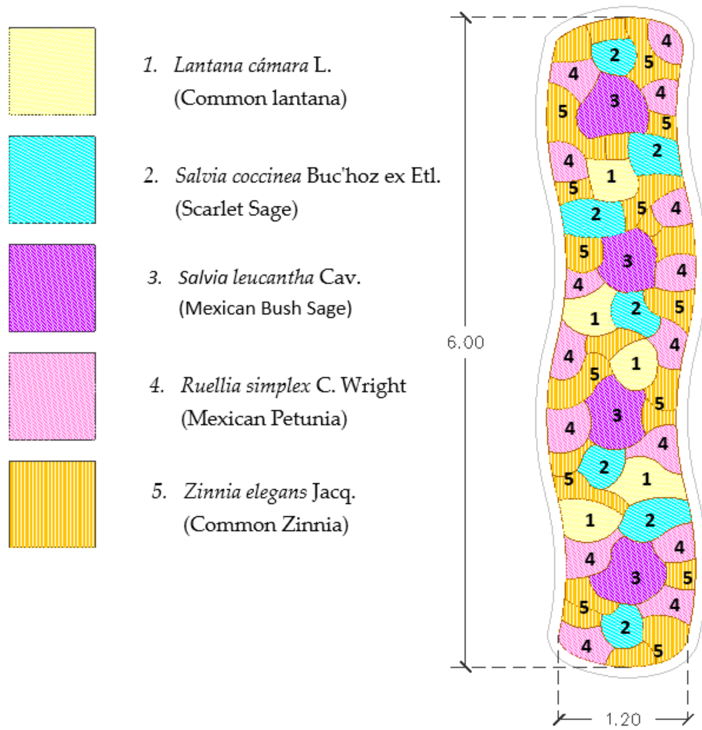

Figure 1. Design and distribution of the plant palette in the naturalistic planting established in Amatlán de los Reyes, Veracruz, Mexico.

25–31, 2018. Before responding, each participant was instructed to stand in front of the planting and observe it for as long as they deemed necessary before answering (García-Albarado and Dunnett, 2009). The level of appreciation was measured on a scale of 1 to 10, with one corresponding to the lowest level of acceptance and 10 to the highest.

Statistical analysis

Statistical analysis was performed using Minitab version 18.1.0, with descriptive statistics, calculation of means, and analysis of variance (ANOVA) to identify significant differences among participants' assessments. The information was classified according to the following variables: color variety, naturalistic appearance, plant diversity, insect diversity, and plant height. Perception assessment was analyzed based on the general characteristics of the visitors, such as age, gender, and development environment (rural or urban). To formally test for statistically significant differences between groups, multiple comparisons were performed using Fisher's method.

RESULTS AND DISCUSSION

The use of ANOVA and *post hoc* tests was adequate for analyzing differences in perception between visitor groups; however, statistical assumptions (normality, homogeneity of variances) were not reported, which constitutes a methodological limitation. The interpretation of the results must be qualified in the case of non-significant differences, as occurred between visitors from rural and urban settings. The absence of statistical differences does not necessarily imply that both groups perceive the planting identically, but rather that, under the conditions and sample size of the study, it was not possible to detect clear contrasts. Future research with a larger number of participants and stricter control of statistical assumptions could provide greater robustness to these types of comparisons.

Perception patterns across age groups

For analysis of the participant profile, age was classified into five groups: 1 = 10–20 years, 2 = 20–30 years, 3 = 30–40 years, 4 = 40–50 years, and 5 = 50–60 years. Group 1 represented 13 % of the total sample, while the remaining groups represented 33, 32, 14, and 8 %, respectively. Regarding the degree of acceptance among the different age groups, significant differences were detected between the means ($p \leq 0.05$) (Table 2).

Table 2. Statistical means of participants' perception ratings of the naturalistic planting across age groups.

Factor	Mean	Standard deviation	*p* value
10 to 20 years	7.73 a	0.819	0.002
20 to 30 years	7.52 a	0.670	
30 to 40 years	8.93 b	0.692	
40 to 50 years	8.82 b	0.558	
50 to 60 years	9.08 b	0.383	

Mean values in columns with different letters are statistically different ($p \leq 0.05$) according to Fisher's method for comparisons.

The results indicated that people over the age of 30 tended to value naturalistic planting more than younger people. This behavior could be explained by the fact that, over time, people develop a greater awareness of conservation and appreciation for nature (García-Albarado and Dunnett, 2009; Müderrisoglu and Gültekin, 2013). Previous research has shown that accumulated experience and prolonged exposure to natural environments positively influence the aesthetic and functional appreciation of the landscape. Older age groups tend to associate naturalistic landscapes with personal memories and experiences linked to the rural environment (Chiesura, 2004). In the case of age, adults tended to rate the naturalistic planting more positively, which was interpreted as possible “greater environmental awareness.” While this explanation is plausible, it should be acknowledged that it constitutes a linear and simplified interpretation. Factors such as educational level, previous experience with gardens, or social status could also influence visitors’ assessments, but these variables were not considered in the study design. Recognizing this methodological limitation strengthens the transparency of the results and opens up the possibility for future research to more accurately understand the factors that shape social perceptions of naturalistic gardens.

Perception patterns across genders

The gender variable in the perception of the planting proved significant, with women showing a greater affinity for the naturalistic design. Of the total number of participants who answered the questionnaire, 54 % were women and 46 % were men, indicating a balanced proportion between genders. The results indicated that women gave higher ratings than men for all the attributes evaluated in the planting, including colors, naturalistic appearance, plant diversity, insect diversity, and plant height (Table 3, Figure 2).

Table 3. Statistical means obtained from the participants’ ratings of the naturalistic planting based on gender.

Factor	Mean	Standard deviation	*p* value
Female	8.79 a	0.819	0.031
Male	7.73 b	0.670	

Means that do not share the same letter denote that they are significantly different using Fisher’s method for comparisons ($p \leq 0.05$).

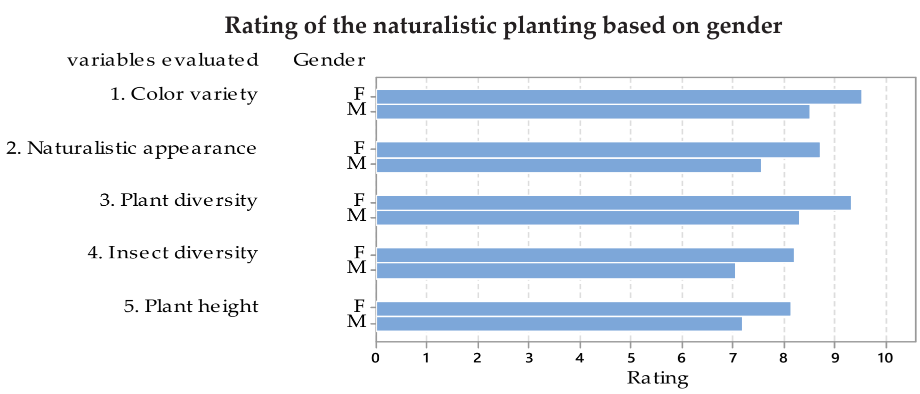

Figure 2. Mean perception ratings of the naturalistic planting variables by gender (1 = lowest rating; 10 = highest).

This pattern is consistent with findings from previous studies indicating that women tend to exhibit greater affinity for green environments and heightened sensitivity toward environmental conservation (Braçe *et al*., 2021). Most female participants stated that, in addition to being attracted to the colors and shapes of flowers, they appreciated the use of wild species in the planting for its contribution to nature conservation. Prior research has shown that women generally show stronger appreciation for the biodiversity, naturalness, and ecological functions of landscapes, whereas men often emphasize functional or recreational aspects (Tindall *et al*., 2003).

Likewise, Strumse (1994) reported that women showed greater acceptance of and sensitivity toward natural environments, rating their preference for ornamental meadows in Norway higher than men. This author links his findings to evolutionary theory, proposing that men were mainly engaged in hunting, while women were responsible for gathering food. This role would have favored the development of a greater awareness of and sensitivity to vegetation, a trait that may persist today. These findings suggest that gender differences in the perception of natural environments could be linked to both sociocultural factors and personal experience with nature.

While some previous studies have interpreted this difference using evolutionary frameworks that connected female vegetation sensitivity to an alleged ancestral role as gatherers (Strumse 1994), this type of explanation reinforces stereotypes and posits a biological determinism that is widely contested in contemporary literature. Recent research in anthropology, archaeology, and gender studies refutes these reductionist views. For example, Anderson *et al*. (2024) demonstrate that women have played an active role in productive activities such as hunting, refuting the idea that they were confined solely to gathering. From this perspective, a more robust interpretation of the results would be to consider that the observed differences respond to sociocultural constructions of gender. Women have historically been assigned roles that involve caring for the home, people, and nature, which may influence them to place a higher value on floral landscapes that are symbolically associated with these spheres.

These results are consistent with previous studies, such as that of García-Albarado and Dunnett (2009), who also found greater acceptance of ecological plantings among women. Similarly, recent research in urban settings (Braçe *et al*., 2021) shows that women value the characteristics of green spaces more highly. Regarding the effect of age, landscape preferences have been documented to vary with experience and cultural memory (Müderrisoglu and Gültekin, 2013), which reinforces the interpretation of the emotional connection of older people with the naturalistic style. In addition, recent findings reveal that the perception of naturalness and biological diversity, such as the presence of birds or plant diversity, increases visitors' mental well-being (Vanhöfen *et al*., 2025), supporting the importance of the visual and ecological attributes valued in this planting.

Influence of urban and rural origin

Of the total number of participants (n = 150), 27 % lived in a rural setting and 73 % lived in an urban setting. Regarding the rural or urban origin of the visitors, the results showed no statistically significant differences ($p \leq 0.05$) in the perception of the naturalistic planting (Table 4). However, the difference in means (8.92 for rural residents versus 8.07 for urban residents) suggests a trend that, although not statistically confirmed in this sample, could be relevant in a more representative study or with a larger sample size. In this regard, it is more accurate to point out that, under the conditions of this study, no significant differences were identified, but future methodological designs could reveal clearer contrasts based on the origin of the visitors.

Table 4. Mean perception ratings of the naturalistic planting based on participants' development environment.

Factor	Mean	Standard deviation	*p* value
Rural environment	8.92 a	0.811	0.096
Urban environment	8.07 a	0.597	

Mean values in columns with different letters are statistically different ($p \leq 0.05$) using Fisher's method for comparisons.

These findings suggest that positive perceptions of naturalistic planting may be mediated by shared cultural, aesthetic, and emotional factors rather than the participants' environment. When assessing the acceptance of landscape designs, it may be more relevant to focus on visual and symbolic attributes that transcend the urban or rural context. The degree of acceptance for the naturalistic planting style was high for both rural and urban residents; this could be explained by the benefits that vegetation offers in both contexts. Most participants recognized that this type of planting contributes to ecological functions, particularly the conservation of wild flora and fauna, and many expressed interest in implementing or promoting naturalistic plantings in public spaces as well as in rural and urban gardens (Figure 3).

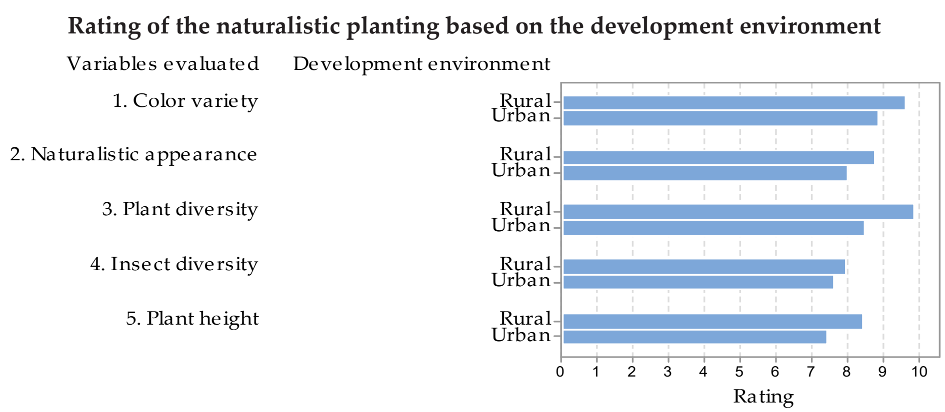

Figure 3. Participants' ratings of naturalistic planting attributes according to their development environment (1 = lowest; 10 = highest).

These findings are consistent with recent studies that observed a convergence in environmental perceptions between urban and rural residents. A systematic review found that more than 50 % of analyses showed no significant differences in the benefits associated with green spaces between urban and rural contexts (Browning *et al*., 2022). Similarly, research on environmental perception discovered that while there are differences in the factors that influence perceptions, such as the community environment in rural areas versus public policies and infrastructure in urban settings, these do not always translate into significant divergences in the valuation of green spaces (Feng *et al*., 2025). According to Mendoza-García *et al*. (2011), rural people have a deep-rooted appreciation for vegetation due to the economic benefits their families derive from it. For urban dwellers, vegetation is essential for health and well-being, in addition to helping to conserve biodiversity (Hoyle *et al*., 2018).

Variables evaluated

Regarding the aspects evaluated in the naturalistic planting, statistically significant differences ($p \leq 0.05$) were found using Fisher's multiple comparisons test. The variables with the highest ratings were "color variety" and "plant diversity" (Table 5). The high ratings for these aspects may be due to the fact that, for most people, visual perception is strongly influenced by color and shape. Both aspects are fundamental to the perception of aesthetics and visual language.

Table 5. Statistical means obtained from participants' general ratings of the variables studied in a naturalistic planting established in Amatlán de los Reyes, Veracruz, Mexico.

Factor	Mean	Standard deviation	*p* value
Color variety	9.07 a	0.934	0.000
Plant diversity	8.85 a	8.853	
Naturalistic appearance	8.20 b	1.220	
Plant height	7.71 c	7.713	
Insect diversity	7.69 c	7.687	

Means that do not share the same letter denote that they are significantly different using Fisher's method for comparisons ($p \leq 0.05$).

These findings are consistent with research that has identified chromatic richness and floral diversity as key attributes for increasing visual appeal and visitor satisfaction in landscaped settings and naturalistic plantings (Lindemann-Matthies *et al*., 2010). A study conducted in the United Kingdom by Hoyle *et al*. (2017) on the emotional responses that can be elicited through the perception of different planting styles showed that most people find a colorful planting with bright flowers preferentially attractive and stimulating. This is consistent with the results obtained in the present study, as the highest-rated variable was "color variety." Lindemann-Matthies and Bose

(2008) demonstrated that aesthetic perception is strongly influenced by the presence of multiple floral colors and is also associated with a higher appreciation of biodiversity. Similarly, Southon *et al*. (2017) found that herbaceous species diversity increases visitor preference, as it is associated with greater naturalness, visual complexity, and ecological potential. From a landscape design perspective, these findings reinforce the importance of selecting species that provide a wide range of colors and floral shapes, as well as structuring plantings with variations in height and texture that generate visual interest over time. Furthermore, alternating flowering periods contribute to maintaining ornamental appeal over several seasons while providing continuous resources for pollinating fauna, which can increase the educational and ecological value of the space (García-Albarado *et al*., 2013).

Integrating aesthetic and ecological criteria into species selection is fundamental to the success of naturalistic plantings in nature tourism contexts. A diverse and chromatically attractive plant palette not only favors social acceptance but also strengthens the role of these spaces as tools for conservation and environmental education. Therefore, taken together, these results suggest that incorporating a diverse plant palette with a wide range of colors can be an effective strategy for improving the social acceptance and ornamental value of plantings oriented toward nature tourism.

Degree of appreciation

Finally, a general analysis was conducted to determine the participants' degree of appreciation for the naturalistic planting style. Of the total number of participants (n = 150) who rated the planting on a scale of 1 to 10, a high level of acceptance was found ($\bar{x}$ = 8.3). The distribution of ratings showed a pattern close to normal, with one corresponding to the lowest level and 10 to the highest level of acceptance (Figure 4).

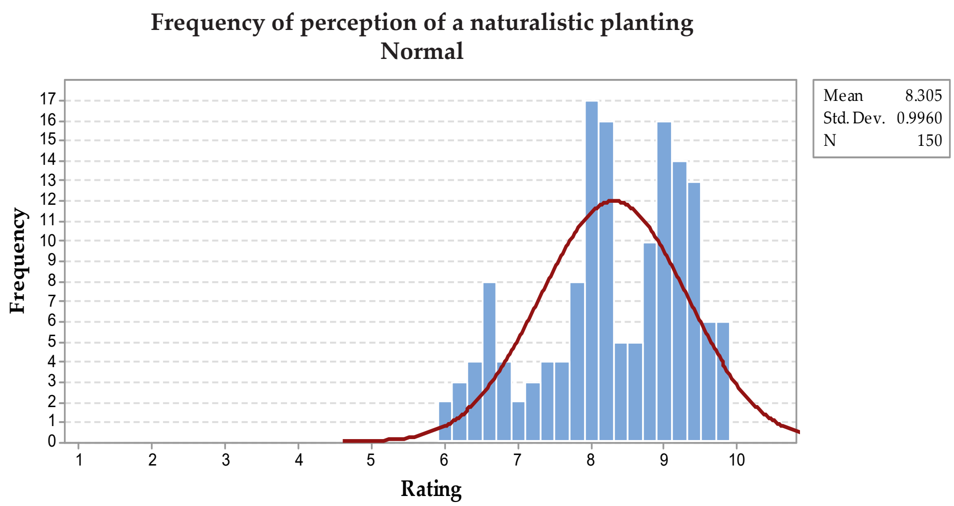

Figure 4. Graph showing the frequency of perception of the naturalistic planting established in Amatlán de los Reyes, Veracruz, Mexico (1 = lowest; 10 = highest).

The high rating indicates that, in general, the naturalistic style of the planting was well received by visitors. When naturalistic plantings present visually attractive attributes, such as color variety, species diversity, and structured composition, their social acceptance tends to increase significantly (Lindemann-Matthies *et al*., 2010; Hoyle *et al*., 2017). Furthermore, the generally positive assessment reinforces the evidence that visitors, regardless of variables such as age, gender, or urban/rural origin, can perceive the aesthetic and ecological benefits of this type of landscape design (Southon *et al*., 2017).

The near-normal distribution of responses suggests homogeneity in perception, which could be attributed to the ability of naturalistic design to generate sensory and emotional experiences shared among different user profiles (Chiesura, 2004). In the context of nature tourism, high social acceptance implies considerable potential for incorporating this type of planting as an ornamental and educational resource, improving the visitor experience and fostering favorable attitudes toward conservation. Furthermore, these results support the recommendation to integrate aesthetic, biodiversity, and ecological functionality criteria into the design of green spaces intended for recreational and tourism use.

Limitations of the study

One limitation of the study was the absence of tourism-oriented questions. Although the planting was developed within an ecotourism context, the questionnaire focused only on aesthetic and ecological aspects. It did not assess their motivation to visit, overall satisfaction, or desire to recommend the site. Considering these variables in future research would allow for better understanding of naturalistic gardens to enhance rural tourism in the region. It is also important to consider whether respondents' perceptions of the associated fauna were based on direct observation or the assumption that they would be present due to the type of plants used. Although pollinators were observed during the experiment, the lack of quantitative records limits the possibility of objectively linking aesthetic perceptions with the actual presence of fauna. Future research could incorporate parallel insect monitoring to link public perceptions with more robust ecological data.

Furthermore, the recorded perception corresponds only to the spring season. Naturalistic gardens, by their very nature, show variations in their composition and attractiveness throughout the year due to the cycles of flowering, growth, and plant succession. For this reason, visitor assessments are likely to change in other seasons, either increasing or decreasing their visual and ecological interest. In future research, it would be advisable to consider inter-seasonal comparisons that allow for a more comprehensive capture of the aesthetic and functional experience offered by this type of planting.

Although the methods employed adequately addressed the stated objective, it is important to recognize the limitation of not having a comparison group. The absence of a control treatment, whether an unplanted area, spontaneous vegetation, or a conventional ornamental design, restricts the ability to contrast visitors' perceptions and more clearly assess the specific contribution of the naturalistic approach. Future research could incorporate comparison groups that would allow for assessing differences in the aesthetic, ecological, and functional acceptance of different design proposals.

CONCLUSIONS

Visitors positively valued naturalistic plantings made up of wild herbaceous species in an ecotourism context. This perception was mainly associated with aesthetic attributes such as color and order, as well as an appreciation of biodiversity, reflecting the potential for this type of design to be socially accepted. This study provides empirical evidence to the debate on naturalistic landscaping in Mexico, showing that plantings with wild species can be understood and appreciated by visitors in rural and urban settings, expanding their potential for application in rural tourism and landscape conservation projects.

Although the methods used met the objective, the lack of a comparison group, the small number of species used, and the evaluation conducted only in the spring season should be noted as limitations. Future research should incorporate greater species diversity, inter-seasonal assessments, tourism variables, and quantitative records of associated fauna to strengthen understanding of the social, aesthetic, and ecological benefits of naturalistic gardens in tropical and subtropical regions of the country.

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PHYSICAL, CHEMICAL, AND BIOLOGICAL CHARACTERIZATION OF SOIL FROM THREE SHADE-GROWN COFFEE FARMS IN JILOTEPEC, VERACRUZ, MEXICO

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ABSTRACT

The soil of shaded coffee plantations in Veracruz, Mexico, provides environmental services such as water regulation, carbon storage, and support for soil biodiversity. However, it has traditionally been treated solely as a physical support for crops, resulting in the overuse of agrochemicals and deterioration of functionality. In light of this problem, this study had the objective of characterizing the physical, chemical, and biological properties of the soil in coffee plantations in the municipality of Jilotepec, Veracruz, to generate useful information for monitoring and promoting sustainable management. Three farms were evaluated: La Barranca, Los Bambús, and San Isidro. La Barranca showed the highest organic matter content (11.87 %), mulch accumulation (57.74 g m^{-2}), and microbial activity (705.7 μg C-CO_2 kg^{-1} d^{-1}), but also had the lowest earthworm density (9.2 individuals m^{-2}), possibly limited by its acidic pH (4.81) and low phosphorus availability (2.04 mg kg^{-1}). Los Bambús farm had a pH value of 5.24, a loamy-clay texture, high microbial respiration (700 μg C-CO_2 kg^{-1} d^{-1}), and the highest earthworm density (17.6 individuals m^{-2}), despite containing a low organic matter content (6.38 %). San Isidro, with a clay texture, had low microbial activity (645 μg C-CO_2 kg^{-1} d^{-1}) and intermediate earthworm density (15.2 individuals m^{-2}), which could be related to its strongly acidic pH (4.09) and low available phosphorus (4.98 mg kg^{-1}). The results reflect the complexity of the edaphic system and the need to consider multiple indicators for integrated soil management that take into account its physical, chemical, and biological properties to improve the sustainability and resilience of coffee systems.

Key words: edaphology, indicators, coffee, management, agroecology.

INTRODUCTION

In Mexico, coffee cultivation (*Coffea arabica* L.) is of great economic importance due to the foreign exchange it generates, as a significant part of production is destined for foreign trade. However, this crop has undergone substantial changes since the implementation of new technologies promoted by the Green Revolution, which, in many cases, have proved ineffective due to inadequate agronomic practices. These conditions have led to growing interest in biodiversity conservation in agriculture, highlighting the role of agroforestry systems in coffee plantations. By incorporating a tree structure that emulates the forest ecosystems where coffee has historically been grown, these systems contribute significantly to the preservation of such environments (Manson *et al*., 2008).

Mexico ranks eleventh in global coffee production, with 710 361 ha destined for this crop (SADER, 2023). Although it is not part of the basic food basket, coffee represents 0.66 % of the national gross domestic product and generates approximately 700 000 direct and indirect jobs. The main producing states are Chiapas, Veracruz, and Oaxaca. In Jilotepec, Veracruz, coffee has been internationally recognized for its high quality, ranking first in the Cup of Excellence competitions in 2017 and 2023. However, there are still few studies focused on soil characterization and the analysis of production practices associated with this crop.

Coffee growers in Jilotepec have faced severe economic losses due to the incidence of pests such as coffee berry borer and rust, which have significantly compromised crop productivity. In response, the use of agrochemicals has increased, which is detrimental to soil health. The soil is a key component of the coffee agroecosystem, providing water and nutrients essential for plant growth through a complex edaphic trophic network (Burbano-Orjuela, 2016; Lavelle, 2021). Understanding the physical, chemical, and biological characteristics of the soil is essential for evaluating its overall functioning. The joint analysis of these properties allows for the diagnosis of its condition, the identification of limiting factors, and the design of appropriate management practices. From this perspective, the objective of this study was to characterize the physical, chemical, and biological properties of the soil in coffee plantations in the municipality of Jilotepec, Veracruz.

MATERIALS AND METHODS

Area of study

The study area is located in the municipality of Jilotepec, in the central mountainous region of the state of Veracruz, Mexico (19° 36′ N and 96° 55′ W), at an altitude between 860 and 1900 m. It borders Naolinco to the north, Banderilla to the south, Rafael Lucio to the southwest, and Tlacolula to the west (Figure 1). The climate is temperate, subhumid, with rainfall in the summer (Cw). Its average annual temperature ranges between 16 and 22 °C, with an annual accumulated precipitation between 1400 and

Figure 1. Location of the study area and sampled farms in the municipality of Jilotepec, Veracruz, Mexico.

1600 mm (INEGI, 2023). The soil is mainly andosol, characterized by volcanic ash and grayish tones (INAFED, 2013).

The area is characterized by a rugged landscape with steep slopes ranging from 10 to 45 % in most of the territory where coffee farming is located (INEGI, 2023). In the municipality of Jilotepec, mesophilic mountain forests, oak forests, and tropical deciduous forests predominate. The main species of flora include *Persea schiedeana* Nees, *Quercus xalapensis* Bonpl., *Fraxinus uhdei* (Wenz.) Lingelsh., *Populus mexicana* Wesm., *Salix humboldtiana* Willd., *Inga vera* Willd., and various ferns, orchids, and bromeliads (Rivera-Hernández *et al*., 2019).

For this study, three coffee farms established more than 25 years ago were selected, all of which are cultivated using the shade-grown system (Figure 2). Coffee plants

Figure 2. Selected coffee farms in the municipality of Jilotepec, Veracruz, Mexico. A: San Isidro; B: Los Bambús; C: La Barranca.

of different varieties are intermingled on the farms, with densities greater than 2500 plants per hectare. The farms are managed using conventional agronomic practices, with the application of agrochemicals for fertilization and control of weeds, pests, and diseases such as rust and coffee berry borer, although some farms also incorporate organic amendments. The geographical location, altitude, variety, and density of the coffee plants, as well as other characteristics of the agroecosystem, are described in more detail (Table 1).

Table 1. Geographic location, size, and characteristics of the study sites in the municipality of Jilotepec, Veracruz, Mexico.

Sites	Latitude Longitude	Size (ha)	Characteristics
San Isidro	19° 36′ 12.15′′ 96° 54′ 44.91′′	2	The dominant coffee variety is 'Costa Rica', followed by 'Mundo Novo' and 'Sachimor' in smaller proportions. Plant density is approximately 2500 plants per hectare. The system includes around 160 shade trees, mainly *Inga* spp. and *Ficus* spp. Management is conventional: horse manure and coffee compost are applied, along with chemical fertilizers once a year. Weed control is performed three to four times annually by mowing, and biological control of the coffee berry borer is implemented. The slope exceeds 30 %.
Los Bambús	19° 36′ 42.7′′ 96° 65′ 16.01′′	1.5	The coffee varieties cultivated are 'Costa Rica' and 'Sarchimor'. Plant density is approximately 2857 plants per hectare. The system includes about 100 shade trees, primarily *Inga* spp., *Citrus* spp., and *Musa paradisiaca* L. Management practices are conventional: chemical fertilizers are applied once a year, and weed control is performed three to four times annually by mowing. The slope of the area exceeds 30 %.
La Barranca	19° 36′ 38.07′′ 96° 55′ 40.57′′	2	The coffee varieties cultivated are 'Costa Rica', 'Typica', 'Mundo Novo', and 'Sarchimor'. Plant density is approximately 2,857 plants per hectare. The system includes about 100 shade trees, mainly *Inga* spp., *Trema micrantha* (Roem. and Schult.) Blume, and *Citrus* spp. Management is conventional: chemical fertilizers are applied once a year, and both compost and leaf mulch are incorporated into the soil. The slope of the area is less than 10 %.

Sampling

Considering that the farms have an approximate area of 2 ha each, according to the Tropical Soil Biology and Fertility (TSBF) method (Anderson and Ingram, 1993), a transect of five monoliths separated by 5 m was delimited. At these points, biological properties were determined from soil samples obtained at a depth of 0 to 30 cm. The collected soil was dried in the shade at room temperature (25–28 °C). Physical and chemical analyses were performed in the laboratory of the Institute of Ecology (INECOL A.C.), following the guidelines established by NOM-021-SEMARNAT-2000 (DOF, 2002).

Physical analysis

For physical analyses of bulk density and texture (Bouyoucos), NOM-021-SEMARNAT-2000 (DOF, 2002) and the Tropical Soils Manual (Moreira *et al*., 2012) were followed. Field capacity was measured using the gravimetric method. Soil samples were saturated in running water and then drained for 24 h. The calculation was performed using the formula:

$$\text{Moisture content} = \frac{\text{Wet weight - Dry weight}}{\text{Dry weight}}$$

Five samples were analyzed at each farm. Tests to determine structure were carried out following the guidelines for visual assessment of soil quality (Noellemeyer *et al*., 2021).

Chemical analysis

Chemical tests included determining organic matter using the Walkley and Black (1934) method and analyzing available phosphorus (P) using the Bray and Kurtz (1945) method. pH and electrical conductivity were measured with a potentiometer in a supernatant suspension of a soil-water mixture (1:2). Electrical conductivity results are expressed in decisiemens per meter (dS m^{-1}). Five samples were analyzed for each farm.

Biological analysis

Biological evaluations included litter weight and earthworm counts, both conducted using the TSBF method (Moreira *et al*., 2012). Soil microbial respiration was measured according to Isermeyer (1952). Earthworm abundance was expressed as the number of individuals per square meter (individuals m^{-2}). Five samples were collected from each farm for evaluation.

Statistical analysis

The assumptions of normality and homogeneity were verified using the Kolmogorov-Smirnov and Bartlett tests, respectively. The data were subjected to a one-way analysis of variance (ANOVA). In cases where significant effects were detected ($p < 0.05$), means were compared using Tukey's test (HSD), using the STATISTICA statistical package version 7.0.

RESULTS AND DISCUSSION

Physical characterization of the soil

In this study, differences in soil texture classes were observed among the three farms sampled: clayey in San Isidro, loamy-clayey in Los Bambús, and loamy-clayey-sandy in La Barranca (Table 2). The percentage composition of soil particles determines soil texture (Porta-Casanellas, 2008), which in turn can significantly influence coffee plant growth and development.

Table 2. Values of physical, chemical, and biological parameters obtained in the farms sampled in the municipality of Jilotepec, Veracruz, Mexico.

Parameters	San Isidro	Los Bambús	La Barranca
	Physical		
Texture	Clay	Clay loam	Sandy clay loam
Clay (%)	44.36 ± 1.10 a	32.36 ± 1.63 b	26.36 ± 1.60 c
Silt (%)	20.0 ± 1.25 b	26.0 ± 1.73 a	20.0 ± 1.39 b
Sand (%)	35.64 ± 2.33 c	41.64 ± 3.45 b	53.60 ± 2.63 a
Bulk density (g cm^{-3})	1.45 ± 0.18 a	1.6 ± 0.08 a	1.32 ± 0.25 a
Structure	Good	Moderate	Good
Field capacity (%)	31.72 ± 1.91 a	22.69 ± 2.15 b	21.62 ± 3.12 b
	Chemical		
Organic matter (%)	9.13 ± 3.1 ab (Medium)	6.38 ± 2.10 b (Medium)	11.87 ± 2.50 a (High)
Available phosphorus (mg kg^{-1})	4.98 ± 0.88 a (Low)	2.67 ± 0.89 b (Low)	2.04 ± 0.82 b (Low)
pH	4.09 ± 0.45 b	5.24 ± 0.70 a	4.81 ± 0.43 ab
Electrical conductivity (mS m^{-1})	0.20 ± 0.05 a	0.09 ± 0.05 b	0.11 ± 0.03 b
	Biologic		
Earthworms	15.2 ± 6.94 ab (Poor)	17.6 ± 5.17 a (Poor)	9.2 ± 4.20 b (Poor)
Mulch (g m^{-2})	46.89 ± 23.83 ab	27.02 ± 10.53 b	57.74 ± 22.43 a
Microbial respiration (µg C-CO_2 kg^{-1} d^{-1})	645.0 ± 4.94 b	700.0 ± 4.94 a	705.7 ± 4.94 a

The data correspond to the average of five repetitions ± standard deviation. Different letters represent significant differences ($p < 0.05$).

The optimal texture for coffee development corresponds to loamy soils, characterized by an adequate balance between sand, silt, and clay (Rosas-Arellano *et al*., 2008). This type of soil allows for good water and nutrient retention while promoting drainage and aeration. The soils in this region are of volcanic origin and have an approximate clay content of 40 %, which gives them a high phosphorus retention capacity, thus reducing its loss through leaching (Sadeghian, 2008). In this context, Los Bambús and La Barranca have textures suitable for coffee cultivation, while San Isidro has a less favorable texture due to its high clay content. This condition can cause drainage and aeration problems, limit root system development, and encourage the proliferation of pathogenic microorganisms (Márquez-de la Cruz *et al*., 2022).

In this study, bulk density was similar between farms ($p < 0.05$), with values ranging from 0.88 to 0.9 g cm^{-3} (Table 2), which is within the optimal range suggested for coffee cultivation (0.8 to 1 g cm^{-3}) by Sadeghian *et al*. (2019). The physical conditions of the soil on the farms are favorable for root development and water infiltration, given that bulk density reflects the mass of soil solids and their pore space in relation to the total volume of undisturbed dry soil (Porta-Casanellas, 2008). This indicator is essential for assessing compaction, porosity, and water retention capacity, which directly affect plant growth and microbial activity in the soil (Salamanca-Jiménez *et al*., 2018).

According to Lince-Salazar (2021), maintaining this bulk density, mainly in the surface strata (0–20 cm), is crucial in areas with steep slopes, such as those in this region, as it promotes adequate rainwater infiltration and helps minimize soil erosion. On the other hand, the results coincide with those reported by Geissert and Ibáñez (2008), who recorded values of 0.8 to 1.4 g cm^{-3} in coffee plantations in Coatepec-Huatusco, Veracruz, showing variations associated with management and topographic characteristics.

The results of soil structure on the San Isidro and La Barranca farms indicate good condition, while Los Bambús was classified as moderate according to Noellemeyer *et al*. (2021) (Table 2). These data are consistent with Geissert and Ibáñez (2008) in a study carried out in the central area of Veracruz (Coatepec-Huatusco), where they observed that forest and coffee plantation soils have a porous and light structure, derived from the three-dimensional arrangement of solid particles and porous spaces that form between them. The authors highlight that the development of this porous structure is the main cause of the low bulk density observed in the volcanic soil characteristic of this region.

Field capacity was significantly higher ($p < 0.05$) at San Isidro (31.72 %) than La Barranca (21.62 %) and Los Bambús (22.69 %) (Table 2). This difference is related to the higher clay content in San Isidro (44.36 %), which favors greater water retention (Lince-Salazar, 2021). Field capacity corresponds to the water content that the soil retains after heavy rainfall or irrigation, once gravitational drainage has ended (24–48 h) and water potential stabilized (FAO, 2024). This is a key indicator of water availability for crops. The values in San Isidro are close to the optimal range of 30–35 % reported by Geissert and Ibáñez (2008) for coffee plantations on volcanic soils in Coatepec-Huatusco, Veracruz, indicating good water availability in the surface profile.

Soil chemical characterization

Organic matter content was 9.13 % in San Isidro, 6.38 % in Los Bambús, and 11.87 % in La Barranca (Table 2). According to NOM-021-SEMARNAT-2000 (DOF, 2002), these values are in the medium to high range. However, according to Sadeghian *et al*. (2019), for coffee cultivation, values below 8 % are considered low, between 8 and 16 % are medium, and above 16 % are high. Therefore, San Isidro and La Barranca have medium levels, while Los Bambús has low content. The variation in organic matter between sites can be attributed to differences in agricultural management, vegetation cover, and soil conservation practices.

The results are consistent with studies in the coffee-growing region of Veracruz. For example, in Tlapacoyan, organic matter contents ranging from 3.74 to 10.09 % were reported. In Colombia, González-Osorio (2019) found organic matter levels ranging from 4 to 7 %, depending on management and geographic location. Adequate organic matter content in the soil plays a crucial role in pH stabilization, as it acts as a mixture of weak acids and allows pH regulation (Sadeghian, 2016). Moreover, it influences soil texture and structure by improving aeration in clay soils and increasing water retention and adhesion in sandy soils, which in turn affects nutrient content and availability (Palma-López *et al*., 2015).

The levels of available phosphorus in the farms evaluated were 4.98 mg kg^{-1} in San Isidro, 2.67 mg kg^{-1} in Los Bambús, and 2.04 mg kg^{-1} in La Barranca (Table 2). According to the classification proposed by Sadeghian *et al*. (2019), values below 10 mg kg^{-1} are considered low, between 10 and 20 mg kg^{-1} are medium, and above 20 mg kg^{-1} are high. Therefore, all three sites have low levels of available phosphorus. In Tlapacoyan, Veracruz, Márquez-de la Cruz *et al*. (2022) reported phosphorus levels in coffee soils ranging from 3.74 to 10.09 mg kg^{-1}, indicating limited availability of this nutrient. In Colombia, González-Osorio (2019) found phosphorus contents ranging from 4 to 7 mg kg^{-1}, also in the low range.

The soils of the three farms showed a pH classified as strongly acidic to moderately acidic (4.09–5.24) (Table 2) according to NOM-021-RECNAT-2000 (DOF, 2002). The ideal pH range for coffee growth is between 5 and 5.5 (Sadeghian, 2016); values outside this range are limiting, either due to acidity (pH <5) or alkalinity (pH >5.5). Acidity restricts the availability of nutrients such as phosphorus and calcium, and when exchangeable aluminum contents exceed 1 cmolc kg^{-1}, toxicity increases, affecting biological activity and crop development.

The electrical conductivity (CE) values in the soils evaluated were 0.2 mS cm^{-1} for San Isidro, 0.09 mS cm^{-1} for Los Bambús, and 0.11 mS cm^{-1} for La Barranca (Table 2). According to FAO (2005), soils with CE values below 0.4 mS cm^{-1} are considered non-saline, a condition that favors coffee growth by preventing salt stress. According to NOM-021-SEMARNAT-2000 (DOF, 2002), the effects of salinity were negligible, with values less than one. Márquez-de la Cruz *et al*. (2022) reported EC values below 0.3 mS cm^{-1}, confirming low salinity. Similarly, González-Osorio (2019) documented similar values in Colombian coffee plantations, highlighting the importance of maintaining low salt levels to preserve soil health and productivity.

Soil biological characterization

The mulch values recorded in this study (27.02 to 57.4 g m^{-2}) (Table 2) are considerably low compared to other coffee systems in Latin America. In Costa Rica, Magaña *et al*. (2004) found that in full sun exposure, the mulch layer stored 240 g m^{-2}, while under shade with *Eucalyptus,* it reached values of 840 g m^{-2}. Although specific literature on mulch biomass in coffee plantations is limited in Mexico, research on agroforestry systems in Veracruz and Chiapas recognizes mulch as an essential component in soil protection and fertility (Paz-Pellat *et al*., 2022), with a key role in carbon and moisture conservation.

The low accumulation of mulch could be due to low tree cover, intensive cleaning practices that remove leaf litter, the use of herbicides, or limited plant biomass production. This deficit has significant ecological implications, as mulch acts as a protective barrier against erosion, regulates soil temperature and moisture, and is a source of resources for edaphic fauna that degrade it and incorporate it into the soil matrix. In this context, the results indicate low functionality of the edaphic system in terms of surface organic cover.

The results obtained from the farms show a very low density of earthworms, with average values between 9.2 and 17.6 individuals m^{-2} (Table 2), well below the value proposed by Geissert *et al*. (2013), who establish that a population is considered rich when it exceeds 900 individuals m^{-2} in coffee soils. This low abundance reflects severe biological impoverishment and could indicate unfavorable soil conditions, such as low mulch production, soil acidification, compaction, or intensive use of agrochemicals.

In a comparative study between coffee plantations and forests in Colombia, Rueda-Ramírez and Varela (2016) found earthworm densities of between 147.2 and 281.6 individuals m^{-2}, suggesting moderate biological activity, but still limited compared to more conserved agroforestry systems, where values close to 900 individuals m^{-2} are reported. This difference suggests that, although other groups of macroinvertebrates may persist, earthworms are particularly sensitive to disturbances in the system, especially native species. In a shade-grown coffee plantation in Cuba, Ferrás-Negrín and Rusindo-Hernández (2025) found an average density of 354 individuals m^{-2} and a biomass of 80.25 g m^{-2}, dominated almost exclusively by the exotic species *Pontoscolex corethrurus*, which is known for its tolerance to disturbed environments. These values far exceed those of this study, highlighting the magnitude of the biological impact on the farms assessed.

Microbial respiration values differed between farms ($p < 0.05$); the highest value was detected in La Barranca (705.7 µg C-CO_2 g^{-1} d^{-1}) and Los Bambús (700 µg C-CO_2 g^{-1} d^{-1}), while in San Isidro, the values were significantly lower (645 µg C-CO_2 g^{-1} d^{-1}) (Table 2). These respiration values indicate high microbial activity, comparable to or higher than those reported in other coffee systems in Latin America. For example, Durango *et al*. (2015) reported values of 580 µg C-CO_2 g^{-1} d^{-1} in coffee plantations in Costa Rica, while Chavarría-Bolaños *et al*. (2012) found 640 µg C-CO_2 g^{-1} d^{-1} in forest systems and 440 µg C-CO_2 g^{-1} d^{-1} in unshaded coffee, indicating that more complex systems with greater vegetation cover promote greater biological activity in the soil.

In contrast, Paolini-Gómez *et al*. (2008) reported considerably lower soil respiration values (15.5–26 mg C-CO_2 kg^{-1} d^{-1}) in traditionally managed Venezuelan coffee plantations established on acidic soils. Although subsequent research (Paolini-Gómez, 2017) recorded increases of up to 76.2 mg C-CO_2 kg^{-1} d^{-1} in organically managed systems, these values are still lower than those observed in the present study. Similarly, Azevedo-Junior *et al*. (2017) reported rates of between 60 and 70 mg C-CO_2 kg^{-1} d^{-1} in Brazilian organic coffee plantations, while Pardo-Plaza *et al*. (2019) documented a range of 18.5 to 37 mg C-CO_2 kg^{-1} d^{-1}.

The data obtained from the three farms studied suggests that La Barranca, with the highest organic matter content (11.87 %), also showed the highest microbial activity (705.7 μg C-CO_2 kg^{-1} d^{-1}) and the highest mulch accumulation (57.74 g m^{-2}). However, it had the lowest earthworm density (9.2 individuals m^{-2}), indicating that resource availability does not necessarily translate into greater earthworm abundance but rather into other conditions, such as acidic pH (4.81) or low phosphorus availability (2.04 mg kg^{-1}), which are limiting factors.

In contrast, Los Bambús, with high microbial respiration (700 μg C-CO_2 kg^{-1} d^{-1}) and moderate mulch (27.02 g m^{-2}), had the highest earthworm density (17.6 individuals m^{-2}). This farm has the highest pH (5.24) and a loamy-clay texture, which may suggest more stable and favorable soil conditions for earthworms, despite its lower organic matter content (6.38 %). On the other hand, San Isidro, with a clay texture and good structure, had lower microbial activity (645 μg C-CO_2 kg^{-1} d^{-1}) and an intermediate earthworm density (15.2 individuals m^{-2}), despite having a high organic matter content (9.13 %). The strongly acidic pH (4.09) and low phosphorus availability (4.98 mg kg^{-1}) could be limiting microbial activity.

CONCLUSIONS

A comprehensive analysis of the soils on all three farms revealed significant differences in physical, chemical, and biological properties. In physical terms, textures ranged from clayey to sandy loam, with good or moderate structures and bulk densities within acceptable ranges, although with differences in field capacity that may affect water retention. Chemically, variable organic matter and carbon contents were identified, as well as generally acidic pH and low phosphorus availability, conditions that may limit biological activity and nutrient availability. Biologically, the soils showed low earthworm density and low humus values, while microbial respiration values were high.

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