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Supplemented culture medium to promote the growth and isolation of *Spiroplasma melliferum*

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Abstract

The study of microorganisms requires controlled conditions that allow their growth outside their natural host, making culture media fundamental in microbiology. These media provide essential nutrients for microbial survival, proliferation, and isolation. Spiroplasmas, are helical and motile bacteria belonging to the class Mollicutes, exhibit a marked metabolic dependence due to the absence of a cell wall and the loss of multiple biosynthetic pathways. For this reason, there *in vitro* culture it is possible to use media supplemented with animal serum and complex extracts, such as LD8 medium and its variants, which supply compounds that these bacteria cannot synthesize. Supplementation of culture media with yeast extract, serum, and cholesterol is necessary to ensure cell membrane integrity, viability, and growth of fastidious microorganisms such as Spiroplasmas. The use of enriched media has enabled the isolation and physiological, genomic, and molecular characterization of Mollicutes, facilitating the understanding of their symbiotic interactions with hosts. However, culturing Spiroplasmas remains a challenge due to their high nutritional requirements. The present study aimed to evaluate different precursors to promote the growth and isolation of *Spiroplasma melliferum.*

Keywords: Culture media; Supplementation; Spiroplasmas; Isolation; Growth

1. Introduction

The study and characterization of microorganisms depend on controlled conditions that allow their growth outside their natural host. Culture media constitute the foundation of microbiology, as they provide the essential nutrients for the survival, proliferation, and isolation of bacteria and other organisms. A culture medium can be defined as a solid or liquid nutrient preparation containing sources of carbon, nitrogen, minerals, vitamins, and growth factors necessary for microbial metabolism [1].

Spiroplasmas are helical, motile bacteria of the class Mollicutes that maintain associations with plants and arthropods. Due to their metabolic dependence and symbiotic lifestyle, Spiroplasmas can only be cultured in media supplemented with animal serum and complex extracts, as is the case with LD8 medium and its variants, which allow for the replacement of biosynthetic deficiencies and the maintenance of stable populations under *in vitro* conditions [2]. Thus, the study of Spiroplasma and its symbiotic interactions with insects depends on culture media and their supplementation, since the success of their isolation, growth, and laboratory experimentation depends directly on the availability of specific nutrients.

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Supplementation of culture media consists of adding compounds that increase nutrient availability or provide essential factors that bacteria cannot synthesize on their own. Among the most common supplements are yeast extract, which provides B vitamins and amino acids, and animal serums, rich in proteins and sterols. Cholesterol is essential for membrane integrity in bacteria without cell walls, as are various metabolic precursors, such as nucleotides and cofactors [3]. These additives not only improve cell yield and viability but are also indispensable for the cultivation of fastidious microorganisms that depend on exogenous nutrients.

Supplemented media have proven relevant for the study of bacteria belonging to the class Mollicutes, a group characterized by the absence of a cell wall, reduced genomes, and a marked dependence on their host. Due to the loss of multiple biosynthetic pathways, these organisms cannot synthesize essential compounds such as amino acids, fatty acids, and sterols, thus requiring specific supplements for their *in vitro* growth [4].

The development of media enriched animal serum, yeast extract, and cholesterol has been fundamental for achieving the isolation of different Mollicutes under laboratory conditions. In this context, microbial growth and isolation in supplemented media have allowed not only the physiological characterization of these bacteria but also the establishment of pure cultures that facilitate their genomic and molecular analysis. Isolation represents a key step in microbiology, as it provides the experimental basis for understanding the biology of the microorganism and its interactions with the host [3]. However, the cultivation of bacteria with reduced metabolism, such as Spiroplasmas, remains a challenge due to their high nutritional requirements. The objective of the study was to evaluate different precursors to promote the growth and isolation of *Spiroplasma melliferum*.

2. Materials and methods

2.1. Culture medium for *Spiroplasma melliferum* ATCC 29416

Preparation of the traditional liquid culture medium (MT) 924-AS4: 1.5 grams of PPLO medium, 0.5 grams of sucrose, 80 ml of distilled water, 0.5 ml of phenol red (0.5%). The pH was adjusted to 7.2, and the mixture was sterilized at 121°C for 15 minutes. Once cooled, 25 ml of horse serum, 10 ml of yeast dialysate, and 0.12 grams of penicillin were added.

Preparation of the agar culture medium (MT) 924-AS4: 1.5 grams of PPLO medium, 0.5 grams of sucrose, 80 ml of distilled water, 1.3 grams of agar. The pH was adjusted to 7.2, and the mixture was sterilized at 121°C for 15 minutes. Once cooled, 25 ml of horse serum, 10 ml of yeast dialysate, and 0.12 grams of penicillin were added.

2.2. Supplemented culture medium for *Spiroplasma melliferum* ATCC 29416

Supplemented medium 1 (A1 and L1): 1.5 grams of PPLO medium, 0.5 grams of sucrose, 80 ml of distilled water, 0.5 ml of phenol red (0.5%). The pH was adjusted to 7.2, and the medium was sterilized at 121°C for 15 minutes. Once cooled, 25 ml of horse serum, 10 ml of yeast dialysate, 1.5 grams of soy protein, and 0.12 grams of penicillin were added. For agar medium, omit the phenol red and add 10 g of agar. Supplemented Medium 2 (A2 and L2): 1.5 g of PPLO medium, 0.5 g of sucrose, 80 ml of distilled water, 0.5 ml of phenol red (0.5%). The pH was adjusted to 7.2 and sterilized at 121°C for 15 minutes. Once cooled, 25 ml of horse serum, 10 ml of yeast dialysate, 1.5 g of Sinoflex, and 0.12 g of penicillin were added. For agar medium, omit the phenol red and add 10 grams of agar.

Supplemented medium 3 (A3 and L3): 1.5 g of PPLO medium, 0.5 g of sucrose, 80 ml of distilled water, 0.5 ml of phenol red (0.5%). The pH was adjusted to 7.2 and sterilized at 121°C for 15 minutes. Once cooled, 25 ml of horse serum, 10 ml of yeast dialysate, 1.5 grams of Hydrasor, and 0.12 grams of penicillin were added. For agar medium, omit the phenol red and add 10 grams of agar.

2.3. Evaluation of the supplemented media

Evaluation of each of the supplemented media and the traditional medium was performed according to the following scheme: 8 ml of each of the media to be evaluated were placed in a container, and 2 ml of the *Spiroplasma melliferum* ATCC 29416 culture were added, with 5 replicates of each. The containers were incubated at 37°C until the pH indicator changed color. Once the media were color-checked, 5 µl were gravity-transferred onto agar plates with 5 replicates and incubated at 37°C. The colony growth of Spiroplasma was monitored using a stereomicroscope (Nikon SMZ-2T Japan). Simultaneously, 200 µl were taken from each of the evaluated media and placed in 96-well microplates with 5 replicates. Five wells free of Spiroplasma cultures (negative control) were included for each of the evaluated media. The pH indicator color change was quantified at 630 nm using a microplate reader (Poweam WHYM201).

2.4. Data analysis

The data from the quantification of colony-forming units (CFU/ml) and the transmittances of the different assays were analyzed using the ANOVA test and its multiple Tukey test with a significance level 0.05 (Instant 2.0 Software)

3. Results

Three supplemented media and the traditional medium were evaluated to monitor Spiroplasma growth. Culture media A1 and L1 (soy protein), both liquid and solid, exhibited turbidity and were therefore discarded. Once the *Spiroplasma melliferum* ATCC 29416 strain was thawed at room temperature, the assays to evaluate the effectiveness of the supplemented media were performed

3.1. Essay 1

At 48 hours, the pH indicator changed color in the liquid media, with the supplemented medium L3 standing out compared to the control and MT (Figure 1).

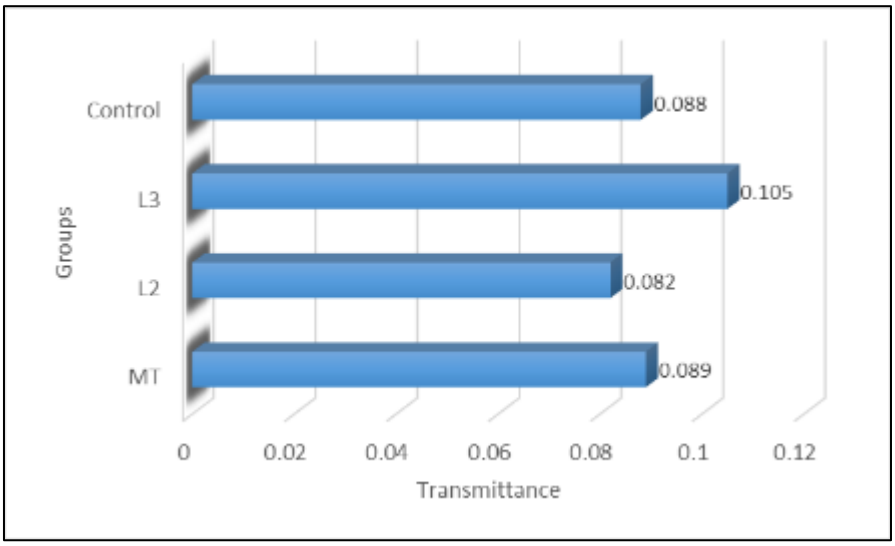

Figure 1 The transmittance readings are shown, revealing a significant difference ($p<0.05$) between group L3 and the rest of the evaluated groups

Five microliters of each of the evaluated samples were subcultured onto agar plates and incubated. After 24 hours of subculture, colonial growth characteristic of Spiroplasmas was observed, with greater growth in the supplemented medium A3, compared to medium A2 and MT (Figure 2).

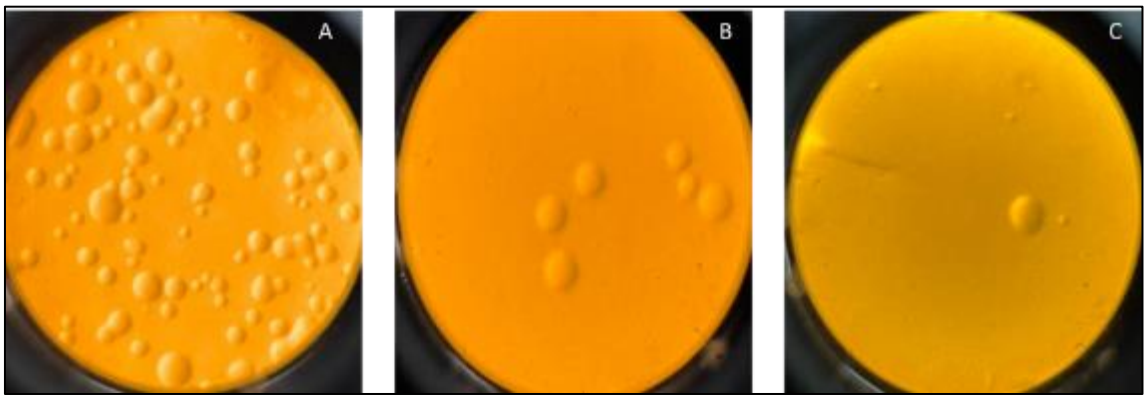

Figure 2 Characteristic fried egg-shaped colonial growth is observed, highlighting greater growth in medium A3 (A), intermediate growth in medium A2 (B) and scarce growth in medium MT (C) (66x)

During the quantification of the pH change values, higher transmittance readings were obtained in the supplemented medium L3 (Figure 3).

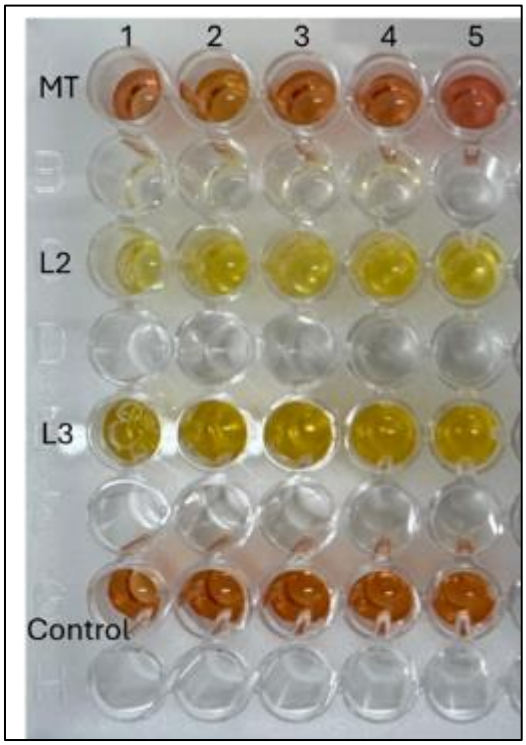

Figure 3 The microplate is shown with its respective samples from each group, where the greater color change is highlighted in the L3 samples

3.2. Essay 2

The pH indicator changed color in the liquid culture media after 24 hours, highlighting that the supplemented medium L3 had a greater change color compared to the control, L2 and MT (Figure 4).

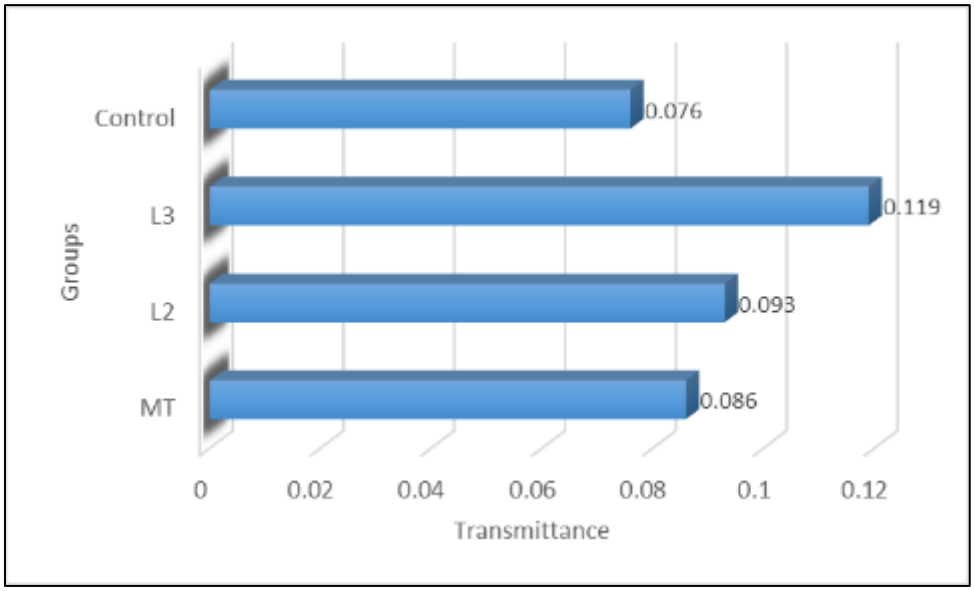

Figure 4 The transmittance values corresponding to test 2 are presented, in which a statistically significant difference ($p < 0.05$) was observed between group L3 and the other groups analyzed

Five microliters of each of the evaluated samples were subcultured onto agar plates and incubated. After 24 hours of incubation, growth was observed, being more evident in the supplemented medium A3 (Figure 5).

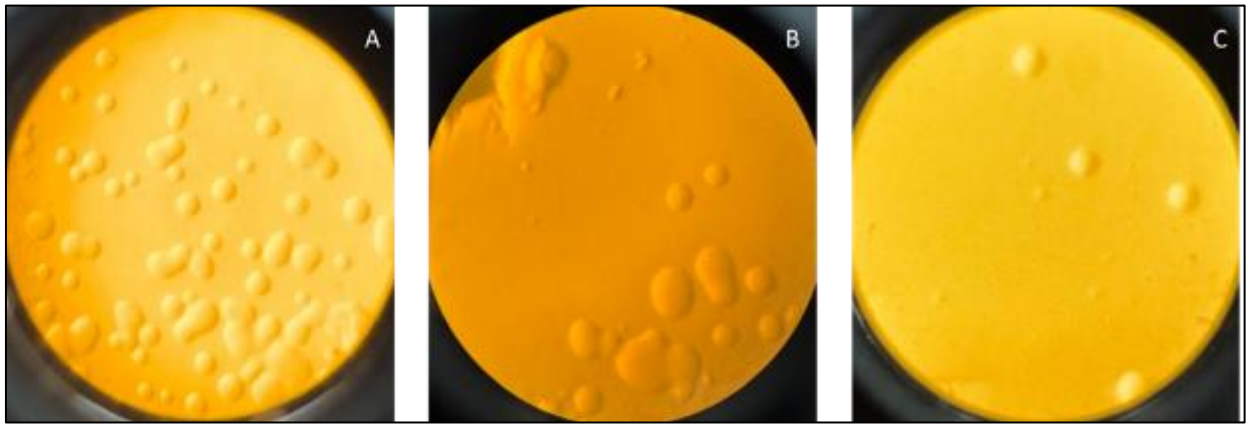

Figure 5 A typical colonial growth pattern with fried egg morphology was evident, with more abundant development observed in medium A3 (A), moderate growth in medium A2 (B) and limited growth in medium MT (C) (66x)

In quantifying the changes associated with the pH change, it was again observed that the highest transmittance values occurred in the supplemented medium L3.

3.3. Essay 3

At 24 hours the pH indicator changed color in the liquid media, confirming that the supplemented medium L3 showed an increase compared to the control, L2 and MT (Figure 6).

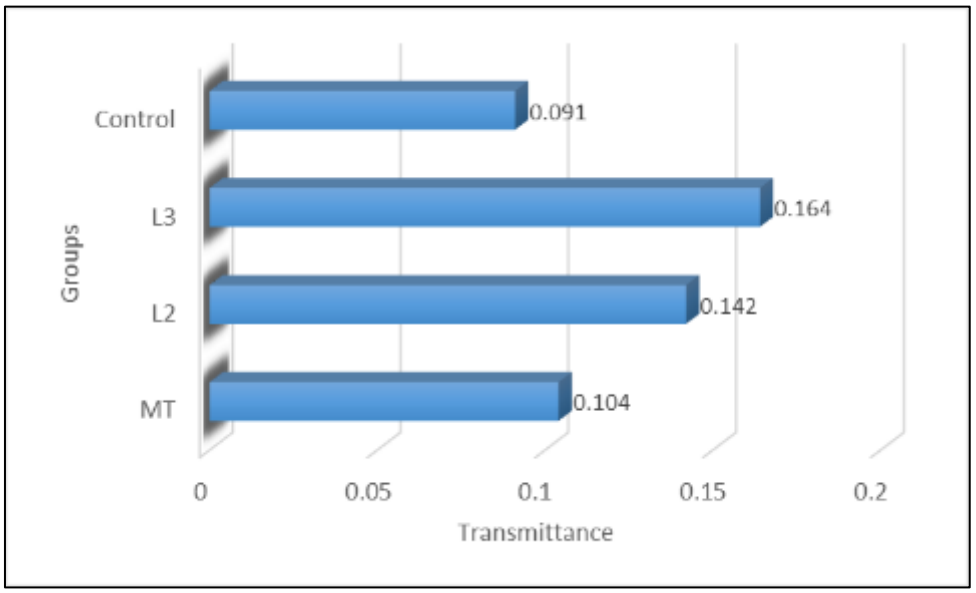

Figure 6 Transmittance values obtained in trial 3, where group L3 showed statistically significant differences ($p<0.05$) compared to the other groups

The culture was subcultured after 24 hours onto 5 µl agar plates of the evaluated media, which were then incubated. After 24 hours, colonial growth characteristic of Spiroplasma was observed (Figure 7).

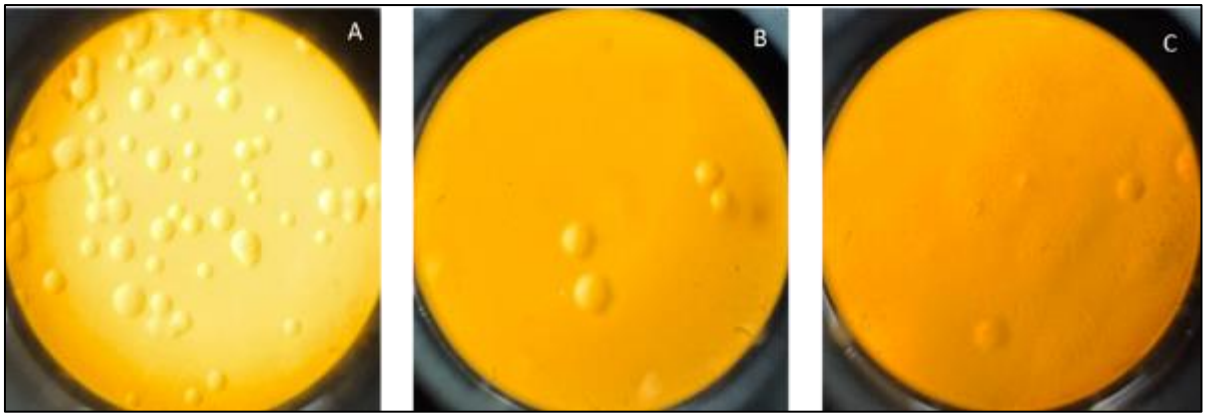

Figure 7 In the samples, a very small amount of colonial fried egg growth was evident in sample A2 and MT (B and C) and abundant growth in A3 (A) (66x)

To quantify the changes linked to the pH change, readings were taken on 96-well microplates, confirming that the highest transmittance values were in the supplemented medium L3.

At the end of the 3 essays and when comparing the data, it was evident that in both the liquid and solid medium of supplement 3, growth was observed in less time and with a higher concentration in CFU/ml, which suggests that the content of supplement 3 (Hydrasor) favored the growth of *Spiroplasma melliferum* ATCC 29416.

4. Discussion

The cultivation of bacteria of the genus Spiroplasma presents a significant methodological challenge due to their physiological characteristics, including the absence of a cell wall, their small genome size, and their marked dependence on exogenous nutrients to sustain their metabolism and cell replication [2-4]. These limitations make the design and evaluation of supplemented culture media essential to ensure not only their survival but also stable, reproducible, and quantifiable growth.

This study comparatively evaluated the performance of three supplemented media against a traditional medium (TM) for the growth of *Spiroplasma melliferum* ATCC 29416, using metabolic, spectrophotometric, and morphological indicators.

One of the first relevant results was the elimination of the media supplemented with soy protein (A1 and L1), which exhibited high turbidity from early stages, in both their liquid and solid forms. This phenomenon can be explained by the complex nature of plant proteins, which contain insoluble fractions, high molecular weight peptides, and colloidal compounds that generate optical interference in liquid media [1].

This turbidity prevents reliable transmittance measurement and hinders the differentiation between actual bacterial growth and medium particles, a critical limitation when working with Mollicutes, whose growth is typically slow and of low cell density [3,5]. Previous studies have indicated that media with complex or poorly defined components can compromise the quantitative evaluation of Spiroplasma growth, recommending the use of chemically defined formulations or those with simple, soluble supplements [5,6].

Once these media were ruled out, the assays focused on supplemented media L2 (Sinoflex) and L3 (Hydrasor), consistently showing that L3 was the treatment that promoted the greatest growth and metabolic activity in all three assays performed. In essay 1, the pH indicator changed color at 48 hours, with significantly higher transmittance values ($p<0.05$) in medium L3 compared to L2, MT, and the negative control. This change reflects acidification of the medium associated with carbohydrate fermentation, a central metabolic process in Spiroplasma, which obtains its energy primarily through glycolysis [2,3].

The presence of anhydrous glucose in the L3 medium formulation is a key factor in explaining these results. Several authors have documented that *Spiroplasma melliferum* exhibits a marked preference for simple carbohydrates, particularly glucose, as its main source of carbon and energy, resulting in rapid organic acid production and an early pH indicator change [3,4,7].

In contrast, L2 medium lacks available carbohydrates, as its composition is primarily based on hydrolyzed collagen, which requires more complex metabolic processes for utilization, thus limiting bacterial growth.

Subculturing on agar plates confirmed that the observed changes in pH and transmittance did indeed correspond to Spiroplasma growth. In assay 1, characteristic colonial growth with a "fried egg" morphology was observed, particularly abundant in A3 medium, while growth in A2 and MT was intermediate and scarce, respectively. This morphology is a distinctive feature of Mollicutes and has been widely described as a diagnostic criterion for the genus Spiroplasma, resulting from the partial penetration of cells into the semisolid agar and their radial expansion [5-7].

The results of essay 2 reinforced the viability of the L3 medium, with the pH indicator changing color at 24 hours, indicating an acceleration of bacterial metabolism. Transmittance values were again significantly higher in L3 ($p<0.05$), suggesting not only greater cell density but also greater metabolic efficiency. The reproducibility of this pattern between consecutive assays is consistent with previous reports, which indicated that the availability of simple carbon sources is a crucial factor for optimizing Spiroplasma growth *in vitro* conditions [5].

Several classic studies have documented that gravity seeding allows Spiroplasma cells to passively settle onto the semisolid agar, facilitating their partial penetration into the medium, a key phenomenon for the development of the typical "fried egg" colonial morphology [5,7]. This morphology results from differential growth: one portion of the cells grows immersed in the agar, while another expands superficially, a process that is severely disrupted when mechanical forces are applied during seeding [6].

Additionally, the ionic composition of medium L3, which includes sodium chloride, potassium chloride, and trisodium citrate, appears to play a role in maintaining osmotic balance. Cell wall-less bacteria, such as Spiroplasma, are highly sensitive to osmotic variations, and an adequate salt concentration is essential for preserving plasma membrane integrity and cell viability [3,6]. It has been noted that an osmotically stable environment favors both cell replication and metabolic expression in these fastidious microorganisms [6].

In essay 3, the results again confirmed the trend observed in previous trials, with significantly higher transmittance values in medium L3 ($p<0.05$). Although medium L2 showed occasional increases in some values, these were not reflected in comparable colonial growth, suggesting that the detected metabolic activity may not be directly related to a sustained increase in viable cells. This discrepancy has been previously described in Mollicutes, where the presence of certain substrates can induce transient metabolic changes without resulting in effective cell proliferation [3,5].

Microscopic observation of colonial growth in assay 3 showed abundant development in A3, while growth was very limited in A2 and MT. These results reinforce the hypothesis that the physiological state of the cells in the liquid medium conditions their capacity for adaptation and expansion in solid media, a phenomenon widely documented in primary isolation studies of Spiroplasma [5,6]. Inoculation of Spiroplasma onto solid media using the gravity technique is a widely recommended methodological procedure for the isolation and cultivation of these microorganisms and is directly related to their structural and physiological characteristics. Spiroplasma belongs to the Mollicutes, a bacterial group characterized by the absence of a cell wall, which confers upon its cells high mechanical fragility and marked sensitivity to external physical forces [3,6,7].

Unlike bacteria with rigid cell walls, Spiroplasma presents only a sterol-rich plasma membrane, responsible for both their helical flexibility and their vulnerability to mechanical stress [3,4]. The application of conventional plating techniques, such as loop or Drigalski spatula inoculation, can cause cell lysis, deformation of helical cells, or loss of viability, drastically reducing the efficiency of primary isolation [5,6]. For this reason, gravity plating by the controlled deposition of small volumes onto the agar surface minimizes physical damage and promotes cell survival.

Additionally, gravity seeding promotes localized cell distribution, which is particularly important considering that Spiroplasma exhibits low initial adhesion efficiency and a limited capacity for rapid colonization of the solid substrate [4,5]. This method increases the likelihood that viable cells will remain in favorable microenvironments, optimizing the establishment of visible colonies after prolonged incubation periods.

The slow growth observed in Spiroplasma compared to other bacteria is due to a combination of genomic, metabolic, and physiological factors, widely documented in the literature. First, Spiroplasma presents reduced genomes, a result of evolutionary adaptations to symbiotic or parasitic lifestyles, which implies the loss of multiple essential biosynthetic pathways [2-4]. Consequently, these bacteria depend almost entirely on the culture medium or the host for obtaining amino acids, nucleotides, lipids, and metabolic cofactors.

This nutritional dependence limits the rate of cell replication, as growth only occurs when adequate nutrients are available at optimal concentrations [3,5]. In suboptimal culture media or those lacking simple carbon sources, such as glucose, energy metabolism becomes inefficient, prolonging the time required to reach detectable cell densities [4,7].

Another determining factor is the relative metabolic inefficiency of Spiroplasma, these bacteria lack a complete respiratory chain and rely primarily on carbohydrate fermentation for energy, a process that generates limited amounts of ATP compared to aerobic respiration [2,3]. This energy restriction results in low growth rates and gradual cell production, which explains why pH indicator change and colony formation typically require 24 to 72 hours, or even longer, depending on the medium used [5,6].

Furthermore, the osmotic sensitivity associated with the absence of a cell wall necessitates maintaining strict ionic conditions in the medium. Minimal fluctuations in salt concentration can induce cellular stress, alterations in the plasma membrane, and decreased viability, further retarding growth [3,6]. This phenomenon explains why media with an adequate electrolyte balance, such as the L3 medium evaluated in this study, promote faster and more consistent growth.

The slow growth of Spiroplasma has also been associated with its natural, predominantly symbiotic or endosymbiotic lifestyle, where selective pressure favors metabolic efficiency and long-term persistence over rapid cell proliferation [4,8]. This evolutionary trait is inevitably reflected in its *in vitro* behavior, where artificial conditions rarely fully replicate the host environment.

Overall, the results obtained indicate that the supplemented L3 medium is a more efficient and reproducible formulation for the cultivation of *Spiroplasma melliferum*, as it promotes greater metabolic activity, early acidification of the medium, and more abundant and consistent colonial growth. These findings are consistent with previous studies highlighting the importance of media rich in simple carbohydrates and with an appropriate ionic balance for the cultivation of Spiroplasma of both plant and animal origin [4,5,7].

Finally, the optimization of culture media for Spiroplasma has not only technical but also biological implications, given that this genus includes species of agricultural, ecological, and evolutionary relevance, associated with plants, arthropods, and vertebrates, including in recent reports related to public health [4-7]. The establishment of efficient media such as L3 can facilitate future studies on physiology, host-symbiont interaction, and pathogenicity, as well as the isolation and maintenance of strains of experimental interest [8,9].

5. Conclusion

Information was gathered regarding the optimal precursors for the growth of *Spiroplasma melliferum.* Three supplemented media were designed and evaluated to promote the growth and isolation of *Spiroplasma melliferum*. Three supplements (soy protein, Sinoflex, and Hydrasor) were evaluated using the traditional medium to optimize the growth and isolation, and the analysis of the data obtained showed that supplemented medium 3 (Hydrasor) was the most efficient for the growth and isolation of *Spiroplasma melliferum*.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors declare that they have no competing interests.

Author's contributions

All authors contributed equally to the conception and development of the work.

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