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Title: Effects of solid and liquid digestate for hydroponic baby leaf lettuce (*Lactuca sativa* L.) cultivation

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Abstract: Digestate was evaluated as an alternative and sustainable growing medium and nutrient solution in the hydroponic cultivation of baby leaf lettuce (*Lactuca sativa* L.). Nine hydroponic combinations of substrate and fertilization (agriperlite + standard solution, agriperlite + liquid digestate, solid digestate + standard solution, solid digestate + liquid digestate, soil + standard solution, peat moss + standard solution; peat moss + liquid digestate, pelleted digestate + standard solution and pelleted digestate + liquid digestate) were tested and compared for the cultivation of baby leaf lettuce, in three different experiments. During the crop cycles yield, as other agronomical and microbiological parameters were investigated. The combination of agriperlite + liquid digestate, solid digestate + standard solution and pelleted digestate + standard solution enhanced plant growth by affecting the root, the shoot, the total dry weight and SPAD parameters, in the all investigated experiments (+32%, +40%, +29%, +17% respectively). Based on the obtained results, digestate represents a sustainable and alternative growing media or nutrient solution for the cultivation of baby leaf lettuce cultivated in hydroponic system.

Ms. Ref. No.: HORTI21401

Title: Effects of solid and liquid digestate for hydroponic baby leaf lettuce (*Lactuca sativa* L.) cultivation

Dear Editor,

here enclosed please find the revised version of the MS by Ronga et al., with track changes, on which all coauthors agree.

We enclose a separate letter with a precise rebuttal of all doubts and question of the reviewers reporting and tracking any change in the revised version of the manuscript.

We want to thank the Reviewers for their work, for appreciating the manuscript and for the suggestions they have given us to improve the work.

Hoping that the manuscript now meets the quality standards of the Journal, and that it can now answer all the concerns of the reviewers,

Kindest regards,

Sincerely,

Domenico Ronga

Reviewers' comments:

Reviewer #1:

Relevant comments:

1. The research topic of scientific work falls within the general scope of the journal.
2. The interpretations of the results are appropriate and justified by the data obtained. The bibliography used in the discussion supports the explanation of the results obtained. The conclusions that emanate from the work are solid.
3. Research contributes highlighted the possible use of digestates as growing media or nutrient solution to grow baby leaf lettuce with high yield and low microbiological contaminations. The digestate represents a sustainable and alternative growing media or nutrient solution for the cultivation of baby leaf lettuce cultivated in hydroponic system

The author must include the following considerations:

- a. in the summary, only mention the factors that explains the treatments

A: Ok, thanks for the comment. According to Referee's suggestion only the factors that explains the treatments were mentioned in the summary

- b. Line 64-65 and Line 67-68. Update information to the year 2016. This is the last update that appears in the FAOSTAT.

A: Correct, thanks for the observation. We modified the information. Lines 64-69.

- c. Line 197. Surely the evaluation of the percentages of germination was made under some international regulations. As for example the ISTA. Cite source used as the basis for evaluations.

A: Thanks for the good observation. The reference was added. Line 216.

I want to congratulate the authors for the level of depth achieved in the work and the type of analysis performed on the results. The work shows statistical solidity, which support the results obtained.

I believe that by incorporating the suggestions made in the present evaluation, the work is suitable for publication.

Reviewer #2: This is an interesting paper dealing with the use of alternative Growing media and nutrient solution for cultivation of lettuce. The exposure of the authors is considerably clear. My view is that the manuscript could be improved significantly when authors explain last findings in research dealing with the use of organic materials as a substrate. They also are invited to update the references dealing with the use of liquid by-products when should be used as a nutrient solution. The conclusion section should be written down again.

Line 75: "agricultural residues" and "dedicated energy crops" concept: are they excluded each other?

A: Thanks for the observation. We modified the sentence. Lines 75-76.

Line 76. Biofuels is in plural, here? Are there other fuels rather than biogas?

A: Ok. Biogas is the only fuel. We modified the sentence. Line 78.

Line 83: WitH.

A: Sorry for the trivial error; we modified the word. Line 85.

Line 85. Please, revise "while, when"

A: Ok; we revised the sentence. Lines 86-87.

Line 97: "circular economy research" instead of "circular economy"?

A: Correct, thanks for the observation. We modified the sentence according to the Reviewer's suggestion. Line 98.

Line 98. "4" is subscript.

A: Sorry for the mistyped; we changed "4" as subscript. Line 99.

Line 107-108. These references are relatively recent...when this is a well-known concept. Can you include other references?

A: Ok; we included other references. Line 109.

Line 140: revise "regimen"

A: Sorry for the trivial error; we modified the word. Line 155.

Line 141: meaning of RCBD.

A: Thanks for the good observation. The meaning of RCBD was added. Line 156.

Line 144. "In the first": in another paragraph.

A: Correct, thanks for the observation. We added a new paragraph according to the Reviewer's suggestion. Line 161.

Line 148. Why not soil + LD in the first and second experiment sets?

A: Thanks for the good point. For this work we only used soil + standard solution (S + SS) as control in the first and second experiment sets. We added this information also in the manuscript. Line 164. However, your suggestion will be considered in future researches.

Line 157. What does mean 20%?

A: We apologise for the typo; we modified the sentence. Line 174.

Line 165. Explain the active compound of the commercial product that acidifies the medium.

A: Correct, thanks for the observation. We added the information. Lines 182-183.

Line 166. Explain "ca"

A: We apologise for the typo; we modified the sentence. Line 184.

Line 168 Explain briefly the system

A: The system was explained. Lines 186-188.

Line 170. Explain the system

A: The system was explained. Lines 189-191.

Line 233. Explain the main methods.

A: The reference was added. Line 253.

All the document. Please, search for all the document and review the use of "trait": what do you mean? Like "treatment"?

A: Correct, thanks for the observation. We changed the word trait with parameter along the manuscript.

Line 291. Substitute dray by dry.

A: We apologise for the typo; we modified the word. Line 311.

Line 419. Again: the 4 of the ammonium formulae should be subscript.

A: Sorry again for the mistyped; we changed "4" as subscript. Line 439.

Line 480 Please, show data

A: The Table S2, containing the requested data, was added. Moreover, the sentence was rewritten. Line 500.

Line 498. Please, include new insights from the bibliography in which it is explained that ammonia, through nitrification, can be transformed into nitrate to avoid these kind of problems (Waste Management 44, 72-81). In general, you should review other articles dealing with the use as a nutrient solution of liquid byproducts. Apart from the previous one: Agricultural Water Management 140, 87-95.

You also have to do more research on recent developments on alternative growing medium production through composting.

A: Thanks for the good suggestions. The reference (Waste Management 44, 72-81) was added. Lines 520-523. We also added other research that worked on the valorisation of the by-products to obtain innovative growing media and nutrient solutions. Lines 127-140.

Conclusions. I find that the conclusions are too general. Please, check the objectives and state appropriate and specific conclusions of your study.

A: The conclusions were rewritten according to the Reviewer's suggestions. Line 530-546.



Highlights:

- Solid digestate might replace the common growing media in soilless cultivation
- Liquid digestate could replace fertilizer in soilless cropping systems
- Pelleted digestate is an interesting growing media for lettuce soilless cultivation
- The use of digestates are a sustainable approach for lettuce soilless cultivation

1 **Effects of solid and liquid digestate for hydroponic baby leaf lettuce (*Lactuca sativa* L.)**
2 **cultivation**

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29 **Keywords:** digestate, lettuce, hydroponics, soilless, fertilizer, sustainability

30

31 Abstract

32 Digestate was evaluated as an alternative and sustainable growing medium and nutrient solution in
33 the hydroponic cultivation of baby leaf lettuce (*Lactuca sativa* L.). Nine hydroponic combinations
34 of substrate and fertilization (agriperlite + standard solution, agriperlite + liquid digestate, solid
35 digestate + standard solution, solid digestate + liquid digestate, soil + standard solution, peat moss +
36 standard solution; peat moss + liquid digestate, pelleted digestate + standard solution and pelleted
37 digestate + liquid digestate) were tested and compared for the cultivation of baby leaf lettuce, in
38 three different experiments. During the crop cycles yield, as other agronomical and microbiological
39 ~~trait~~parameters were investigated. The combination of agriperlite + liquid digestate, solid digestate
40 + standard solution and pelleted digestate + standard solution enhanced plant growth by affecting
41 the root, the shoot, the total dry weight and SPAD parameters, in the all investigated experiments
42 (+32%, +40%, +29%, +17% respectively). ~~Regarding the nitrate content and the aerobic mesophilic~~
43 ~~charge all the samples were below the threshold for the market (2500 mg kg⁻¹ and 5.0E+05 CFU g⁻¹~~
44 ~~of fresh weight product, respectively).~~ Based on the obtained results, digestate represents a
45 sustainable and alternative growing media or nutrient solution for the cultivation of baby leaf lettuce
46 cultivated in hydroponic system.

47

48

49

50 Abbreviations

51 **Abbreviations:** AD – anaerobic digestion, AG – agriperlite, S – soil, SD – solid digestate, LD –
52 liquid digestate, PD – pelleted digestate, PM – peat moss, VE – vermiculite, NS – nutrient solution,
53 GM – growing media, SS – standard solution, MAC – mesophilic aerobic charge, MAC_{GMC} –
54 mesophilic aerobic charge of growing media and nutrient solution combination, MAC_L –
55 mesophilic aerobic charge of lettuce, SFC = spore forming charge, SFC_{GMC} – spore-forming charge
56 of growing media and nutrient solution combination, CC_L – coliform charge of lettuce, HI – harvest
57 index, RDW – root dry weight, SDW – shoot dry weight, TDW – total dry weight.

58

59 1. Introduction

60 The consumption of fresh-cut vegetables (including herbs) increased over the last 20 years in the
61 European market, at the annual growth rate of about 4%; this is why food category is recognized to
62 be as one of the most profitable in the fruit and vegetables segment. As the result of an upward trend
63 observed during the last decade, lettuce (*Lactuca sativa* L.) and chicory (*Cichorium intybus* L.) are
64 cultivated on a total area of ~1.2 M ha worldwide, with ~275 M t of global production (FAOSTAT,
65 20164). Italy ranks ~~foursixth~~ in the world, with open-field lettuce and chicory covering a total area
66 of ~~3815,54210~~ ha (~~31.715.2~~% in the North, ~~10.01.4~~ % in the Centre and ~~58.373.4~~% in the South),
67 and a total production of about ~~83.15~~ M t (AGRISTAT, 20164a). Greenhouse production is also
68 relevant, with a total area of 4, ~~549264~~ ha (~~37.323.2~~% in the North, ~~31.95.0~~% in the Centre and
69 ~~30.841.8~~% in the South) (AGRISTAT, 20164b). Alongside their wide market spread, leafy
70 vegetables are considered the group of fresh foods with the highest concern for microbiological
71 hazards. Among them, fresh-cut lettuce is frequently linked with food borne outbreaks (López-
72 Gálvez et al., 2010); specifically, the bacterium *Escherichia coli* O157:H7 was found to be strongly
73 associated with lettuce contamination (Franz et al., 2008). Therefore, fresh-cut vegetables might
74 have a relatively short shelf-life which usually does not exceed 6–9 days.

75 Anaerobic digestion (AD) – or co-digestion – is a widely used process to treat various kinds of raw
 76 biomasses, ~~ranging from organic wastes to agricultural residues and dedicated energy crops~~. The
 77 main goal of this technique is to efficiently convert a low-value feedstock into more bio-based
 78 products and renewable biofuels, such as biogas. Besides that, reducing the dependence on fossil
 79 raw materials, AD has the advantage to limit odours and pathogens' charge of the remaining by-
 80 product, technically called digestate (Hijazi et al., 2016; Jolánkai et al., 2014; Nkoa, 2014; Uddin et
 81 al., 2016). As a consequence of the microbial activity that takes place during the AD, digestate
 82 enriches in nutrients already available in the feedstock – scientifically called ingestate – acquiring
 83 the following characteristics: low dissolved oxygen level, high levels of chemical and biological
 84 oxygen demand, rise in its content of suspended solids (Dosta et al., 2007). When compared
 85 ~~with~~ solid digestate (SD), the liquid phase by-products (liquid digestate, LD) are characterized
 86 by lower levels of dry matter, total organic carbon (TOC), C/N ratio and viscosity; ~~instead~~ ~~while~~,
 87 when compared with the ingestate, the liquid digestate ~~is compared with the ingestate, it~~ shows
 88 higher pH and ammonium percentages (Nkoa, 2014; Tambone et al., 2010).
 89 Previous studies have shown that digestate contains phytohormones – above all, gibberellins, indole
 90 acetic acid, auxin-like and auxin-active molecules – dissolved in the organic matter, and other
 91 bioactive compounds that have the potential to promote plant growth, increasing the tolerance to
 92 biotic and abiotic stresses (Liu et al., 2009; Scaglia et al., 2017; Yu et al., 2010). Nevertheless,
 93 antithetical results about the phytotoxicity of digestate were reported in literature: several authors
 94 confirmed that digestate caused phytotoxic reactions (Abdullahi et al., 2008; Poggi-Varaldo et al.,
 95 1999; Salminen and Rintala, 2002). Other studies highlighted positive effects on germination and
 96 growth (Gell et al., 2011; Ronga et al., 2016; Sánchez et al., 2008;); in addition, a very recent report
 97 by Scaglia et al. (2017) suggested the use of digestate as an innovative bio-stimulant to increase the
 98 added value of the AD, positively reinforcing the circular economy research. The phytotoxicity of
 99 digestate after field application is related to the presence of ~~NH₄⁺-N~~ ~~NH₄⁺-N~~ and organic acids;

100 however, no data about the duration of the phytotoxic effect in field conditions have been reported
 101 (Möller and Müller, 2012). The distribution of digestate in the soil might potentially spread both not
 102 pathogenic and pathogenic bacteria (i.e. *Salmonellae*, *Clostridia* and *Listeriae*) that may survive
 103 after the AD (Bonetta et al., 2011; Bonetta et al., 2014; Sidhu and Toze, 2009), causing soil and crop
 104 contamination (Bonetta et al., 2014). To overcome this inconvenient, Pulvirenti et al. (2015)
 105 demonstrated that the pelleting treatment of digestate can be a feasible solution for the elimination
 106 of any microbiological risk.

107 Modern protected horticulture recently shifted from soil-grown systems to soilless one (Martínez et
 108 al., 2013). Soilless systems might support efficient and intensive plant production (Barrett et al.,
 109 2016; [Grafiadellis et al., 2000](#); [Raviv and Lieth, 2008](#); [van Os, 1999](#)). Soilless growing media (GM)
 110 adopted in horticulture normally include both organic (e.g. peat moss) and inorganic (e.g.
 111 vermiculite, rockwool, perlite and/or sand) substrates.

112 Soilless media, fertilizer, irrigation, chemicals and greenhouse structure involve different level of
 113 fossil fuel inputs (Enoch, 1978; Stanhill, 1980). Moreover, the selection of substrate as growing
 114 medium is based on both agronomic performance and economic considerations (Barret et al., 2016).
 115 Peat moss (PM) is one of the most used organic component for the preparation of growing media,
 116 due to its agronomic, hydrological and physic-chemical characteristics (Herrera et al., 2008).
 117 However, peat moss is a non-renewable resource which is turning to be increasingly scarce and,
 118 when available, expensive; in fact, there is a lot of concern about the economic and environmental
 119 impacts related to the exploitation of peatland ecosystems, moreover resulting in fossil CO₂
 120 mobilization (Schmilewski, 2008). In addition, peat-based substrates cause reduction of wetlands
 121 and loss of soil organic carbon (Carlile and Coules, 2013). Hence, the concern on the environmental
 122 impacts of some commonly used materials, such as peat-based growing media and chemical
 123 fertilizers, led researchers to identify and assess new environmental friendly products (Wallach,
 124 2008). Another perspective to be considered is the request of sustainable products, by consumers

(Gül et al., 2007a; Gül et al., 2007b). So that, alternative substrates and nutrient sources for soilless horticulture need to be investigated in a perspective of circular economy and environment preservation (Herrera et al., 2008; Ronga et al., 2016). Interesting previous works assessed the valorisation of the by-products as innovative growing media and nutrient solutions. Gattullo et al. (2017) showed the suitability of a municipal solid waste compost (MSWC) and a sewage sludge compost (SSC) as components of growing media for the soilless cultivation of lettuce. The use of composted agro-waste as growing media might be an efficient alternative to peat-based substrates for controlling diseases, also in soilless production (De Corato et al., 2016). Regarding nutrient solutions by-product management from cheese industry can be a sustainable solution for the irrigation of horticultural crops, such as tomato (Prazeres et al., 2014). Most crop nutrients might be derived from aquaculture (Tyson et al., 2011). Inf act, interesting studies showed the potential for crops to use the nutrient by-products of aquaculture as a nutrient solution (Adler et al., 1996, 2000; Lin et al., 2002). As reported above, there are few published studies on the effects of the digestates on hydroponic production. Therefore, further studies on the use of the digestates on hydroponic production could be very useful to increase agricultural sustainability. The aim of the present study was the evaluation of digestates as sustainable alternative growing media and nutrient solutions for baby leaf lettuce cultivation in hydroponic system. Accordingly, multiple experiments in controlled (growth chamber) conditions were set up, and the effects on yield together with other agronomic and microbiological ~~trait~~parameters were investigated to compare solid and liquid digestate with conventional growing media and nutrient solutions.

2. Material and methods

2.1. Plant material, experimental design and growing conditions

A baby leaf lettuce Batavia blonde type cultivar ‘Chiara’ (Isi Sementi S.p.A., Fidenza, Italy) was selected for cultivation. The genotype is a well-adapted fresh-cut lettuce characterized by a

medium-short growing cycle (20-25 days), with tight erect blonde leaves, and high tolerance to tip burn. Lettuce ‘Chiara’ was sown into separated hydroponic discontinuous closed systems in three independent experiments (crop cycles) conducted in controlled conditions in a growth chamber of the University of Modena and Reggio Emilia (Reggio Emilia, Italy). Plants were grown under long-day conditions (15 h light, 9 h dark; light intensity $180 \mu\text{mol m}^{-2} \text{s}^{-1}$). Relative humidity was maintained at 65%, while temperature regime of the growth chamber varied in the different crop cycles. Each experiment was set up as a randomized complete block design (RCBD) factorial design with 16 replicates (corresponding to 16 pots with 20 lettuce seedlings each) per treatment. In total, five types of solid substrates (PM – peat moss, AG – agriperlite, S – clay-loam soil, SD – solid digestate, PD – pelleted digestate) were combined with two nutrient solutions (SS – standard solution, LD – liquid digestate).

In the first crop cycle the growing temperature was kept at $24 \pm 2 \text{ }^{\circ}\text{C}$ and the following growing media and nutrient solution combinations were tested: agriperlite + standard solution (AG + SS); agriperlite + liquid digestate (AG + LD); solid digestate + standard solution (SD + SS); solid digestate + liquid digestate (SD + LD); soil + standard solution (S + SS), used as control. In the second crop cycle, the growing media and nutrient solution combinations were the same whereas the growing temperature was set at $27 \pm 2 \text{ }^{\circ}\text{C}$. In the third crop cycle the growing temperature was kept at $24 \pm 2 \text{ }^{\circ}\text{C}$ and the following growing media and nutrient solution combinations were tested: peat moss + standard solution (PM + SS); peat moss + liquid digestate (PM + LD); pelleted digestate + standard solution (PD + SS); pelleted digestate + liquid digestate (PD + LD). All crop cycles had the same duration of 21 d.

2.2. Characteristics of the growing media and hydroponic cultivation

Solid substrates used as potting media in this study had the following technical characteristics: agriperlite (AG) Agrilit[®] 3, Perlite Italiana s.r.l. (Italy) – grain diameter 2–5.6 mm, pH 7.5, density 90 kg m^{-3} ~~$\pm 20 \%$~~ , and EC $0.1 \text{ dS} \cdot \text{m}^{-1}$; soil (S) – clay-loam type, organic matter (1.5 %), total N (1.0

175 ‰), pH 8.0 and EC 0.03 dS·m⁻¹. Solid digestate (SD) and pelleted digestate (PD) were obtained in
 176 an AD plant of the Reggio Emilia area (see below). Peat moss (PM) technic[®], Free Peat B.V. (The
 177 Netherlands) – organic C (23 ‰), organic N (0.5 ‰), organic matter (46 ‰), pH 7.4, and EC 0.2
 178 dS·m⁻¹. Finally, to facilitate lettuce germination, a Vermiculite (VE) Saint-Gobain PPC S.p.a. (grain
 179 diameter 0.5–4.0 mm, pH 8.0, density 105 kg ± 15 % g m⁻³) layer was added to all substrates.
 180 The standard nutrient solution (SS) was prepared dissolving ca. 30 g of Hydrofood KB, Scott[®] s.r.l.,
 181 Treviso (Italy) – NPK(Mg) 17-16-11-(2) plus micronutrients fertilizer – in 20 L of distilled water
 182 with the addition of 20 mL of acidifying agent CIFOVIR 1 (N 5%, P₂O₅ 17%, pH 2.5 and EC 2.5
 183 dS m⁻¹), ©CIFO s.r.l., San Giorgio di Piano, (Italy). The liquid digestate (LD) solution was prepared
 184 by mixing ~~ea.~~ 1.25 L of LD with 18.75 L of distilled water and 20 mL of CIFOVIR 1.
 185 A modified Wilma 16 pots hydroponic closed system Atami[®] B.V., Rosmalen (The Netherlands)
 186 was used; briefly the system consisted in a plastic tank (120 cm x 60 cm x 30 cm) containing the
 187 nutrient solution and the pump. The tank was completely covered with a plastic tray that supported
 188 the pots. The plastic tray has a hole suited to draining the nutrient solution. The dimensions of the
 189 pots were 100 mm and 70.7 mm (diameter and height, respectively). A pump Wave STREAM 700,
 190 IDROPONICA[®], Player s.r.l., Roma (Italy) was used to pump-facilitate the NS recirculation and
 191 irrigation. ~~The and pots plants~~ were irrigated with the nutrient solution every 185 min using a low-
 192 flow dripper (2 L h⁻¹) for 5 minutes, apart S that was irrigated two times every day. The two NS
 193 were analysed on a daily basis with a portable multi-parameter instrument HI9813-6, Hanna
 194 Instruments[®] s.r.l., Padova (Italy) to evaluate temperature (°C), turbidity (ppm), EC and pH. Once a
 195 week, 20 l of each NS were replaced to maintain N content, EC and pH within appropriate ranges
 196 (260 – 290 mg L⁻¹, 2.0 – 2.5 and 6.0 – 6.6, respectively). Finally, one week before the harvest, each
 197 experiment was irrigated using only tap water.

2.3. Digestate production and properties

Digestate was produced in an AD plant owned by CAT–Cooperativa Agroenergetica Territoriale, Correggio, Reggio Emilia (Italy) as described by Pulvirenti et al. (2015). After solid/liquid separation of the fresh digestate, the chemical parameters of the liquid digestate were: TOC (3.74%), nitrogen (N 0.34%), potassium (K₂O 0.95%), EC 1.07 dS·m⁻¹, and pH 8.03 (P₂O₅ was completely absent). Conversely, solid phase digestate showed: TOC (17.02%), nitrogen (N 0.74%), phosphorus (P₂O₅ 0.60%), potassium (K₂O 0.76%), EC 0.23 dS·m⁻¹, and pH 8.11. A small fraction of SD was also dried and pelleted accordingly to Pulvirenti et al. (2015), and PD contained: TOC (16.32%), nitrogen (N 0.93%), phosphorus (P₂O₅ 1.94%), potassium (K₂O 1.94%), EC 4.17 dS·m⁻¹, and pH 8.28.

2.4. Phytotoxicity test, microbiological analyses and agronomic ~~trait~~parameters

To evaluate the influence of the different growing media, of the nutrient solutions, and their combinations on lettuce's germination rate, a phytotoxicity test was performed following Zucconi et al. (1981). Briefly, 4 ml of each growing media water extract (50 g l⁻¹), of the nutrient solutions, and their combinations, plus a control treatment of only water were added to Petri dishes containing Whatman filter paper. Three replicates of 20 seeds were prepared, and the plates were incubated 36 h at 25 °C in a Binder ED53, Tuttlingen (Germany) heating chamber. The number of germinated seeds and the average length of roots were derived in order to calculate a Germination Index (GI%)

according to the following formula (Tiquina and Tam, 1998):

$$GI\% = 100 \times (G_t / G_c) \times (R_t / R_c)$$

where,

G_t = number of germinated seeds of the treatment;

G_c = number of germinated seeds of the control;

R_t = average length (mm) of roots of the treatment;

R_c = average length (mm) of roots of the control.

Microbiological analyses were performed separately on the growing media, the nutrient solution, their combinations and baby leaf lettuce, as follows. Regarding the growing media (AG, SD, PM, PD, S), the NS (LD, SS) and their combinations, it was analysed the mesophilic aerobic charge (MAC) and the spore-forming charge (SFC) by mixing 10 g of each sample with 90 mL of peptone physiological solution in a sterile blender bag. The samples used for the determination of *Clostridium spp.* were thermally pre-treated (95 °C for 10 minutes) to activate the spores. Appropriate dilutions of the suspensions were plated onto Petri dishes and incubated at 30 °C for 24 h for MAC; in the case of SFC, the plates were incubated at 30 °C for 48 h in an anaerobic environment. The media used for the enumeration of MAC was Brain Heart Infusion Agar (BHIA, 70138, Sigma-Aldrich) and *Clostridium* reinforced agar (CM0149, Oxoid) for the spore-forming charge. Every sample was plated twice, and the test was repeated five times.

Bacterial charge was calculated after the incubation time according to the formula:

$$\Sigma c / [(n1 + 0.1 n2) d]$$

Where:

Σc = sum of the number of total colonies;

n1 = number of plates used for the first dilution;

n2 = number of plates used for the second dilution;

d = dilution factor of n1.

As far as mesophilic aerobic charge (MAC_L), the *Coliform* charge (CC_L) was analysed as well in lettuce. The microbiological analyses of the baby leaf lettuce were performed as described: 25 g of samples were mixed with 225 ml of sterile peptone water using a stomacher bag. Appropriate dilutions of the solution formed were inoculated inside BHIA media, accordingly with the microbiological protocol: ISO 4833-1:2013 to determine the MAC_L. The same dilutions were also inoculated on Violet Red Bile Agar (VRBGA, CM0485, Oxoid), accordingly with the microbiological protocol: ISO 4831:2006, to determinate the Coliforms load (CL_L). Every dilution

248 was plated twice; the test was repeated 5 times. Bacterial charge was calculated after the incubation
249 time, as cited above.

250 At the end of each crop cycle the following agronomic ~~trait~~parameters were recorded. Before the
251 harvest, plant height (H) was measured, and chlorophyll content was estimated by measuring three
252 leaves by using SPAD-502, Minolta (Japan). A subsample of each treatment was used to detect leaf
253 nitrate content (UNI EN 12014-2:1998) as suggested by Merusi et al. (2010). Shoot (SDW), root
254 (RDW) and total dry weights (TDW) were measured after desiccation in stove at 65 °C. Harvest
255 index (HI), fraction of biomass to root (FTR) and SDW/H ratio were calculated.

256 2.5. Statistical analysis

257 Factorial ANOVA was performed with GenStat 17.0th edition and factors' means were compared
258 using Duncan's test at P<0.05 level. PCA models were used for biplots generation (Jackson, 1991;
259 Wold et al., 1987), and since the considered variables had different scales, a pre-processing auto-
260 scaling step was performed before calculating the PCA.

261 3. Results

262 Hydroponic cultivation might be one of the technical solutions to respond to the increasing demand
263 of food, without the exploitation of new land, especially in the system of vertical (indoor) farms.
264 However, alternative GM and NS are needed to improve the sustainability of traditional soilless
265 cropping system that nowadays uses non-renewable substrate and nutrient solution.

266 Another important variable in the hydroponic cultivation is the temperature that plays a
267 fundamental role on both the crop growth and the microorganism charge. Hence, in the present
268 study the effects of different GM, NS and growth temperature were investigated in different
269 experiments. In the first two experiments, the same variables were assessed, apart from temperature
270 that was set at 24 ± 2 °C and 27 ± 2 °C, in the first and second crop cycles, respectively. Moreover,
271 in the third experiment the effects of PM vs PD were tested using the same NS assessed in the first
272 and second crop cycles.

273 3.1. Microbiological quality of GM and NS and their combinations

274 The microbiological analysis was performed on each GM and NS investigated (Table 1). The
 275 average values of MAC and SFC charges in term of CFU were $1.2\text{E}+06 \text{ g}^{-1}$ and $1.6\text{E}+05 \text{ g}^{-1}$,
 276 respectively. Solid digestate highlighted the highest charge of MAC ($8.3\text{E}+06 \text{ CFU g}^{-1}$) showing a
 277 content seven times higher than the general average, followed by PM ($6.4\text{E}+05 \text{ CFU g}^{-1}$), while SS
 278 highlighted the total absence of MAC charge. Regarding another important microbiological
 279 parameter, such as the SFC charge, CFU values ranging from the total absence on one side, to
 280 $7.3\text{E}+05 \text{ CFU g}^{-1}$ on the other; LD showed the highest value of SFC ($7.3\text{E}+05 \text{ CFU g}^{-1}$) with a
 281 content four times higher than the general average, followed by SD ($5.5\text{E}+05 \text{ CFU g}^{-1}$).

Medium and nutrient solutions ^a	MAC (CFU g ⁻¹)	SFC (CFU g ⁻¹)
SD	8.3E+06 a	5.5E+05 b
PM	6.4E+05 b	0 c
S	5.2E+05 c	1.3E+04 c
AG	5.2E+03 d	1.1E+03 c
LD	7.6E+03 d	7.3E+05 a
PD	1.5E+03 d	1.1E+03 c
SS	0 d	0 c
VE	1.1E+03 d	2.3E+02 c
Average	1.2E+06	1.6E+05

Table 1. Microbiological quality of the growing media and nutrient solutions. Means followed by the same letter do not significantly differ at $P < 0.05$. AG = agriperlite; LD = liquid digestate; PD = pelletized digestate; PM = peat moss; S = soil; SD = solid digestate; SS = standard solution; VE = vermiculite; MAC = mesophilic aerobic charge; SFC = spore forming charge. See text for details.

282
 283 Nevertheless, the combination of GM and NS plays an important role on the contamination of
 284 cultivated crop. Hence, the mesophilic aerobic and spore forming charges due to the combinations
 285 of GM and NS are reported in Table 2. In the first and second crop cycle, the same combinations
 286 were used and SD + LD recorded the highest charges of both MAC_{GMC} and SFC_{GMC} ($3.6\text{E}+06 \text{ CFU}$
 287 g^{-1} and $1.3\text{E}+05 \text{ CFU g}^{-1}$, respectively) sowing ca. a double charges compared to the general

average, respectively; while the combination of AG + SS showed the lowest ones ($7.4\text{E}+05 \text{ CFU g}^{-1}$ of MAC_{GMC} and $3.0\text{E}+03 \text{ CFU g}^{-1}$ of SFC_{GMC}) (Table 2A). As far as the different combinations investigated in the third crop cycle (Table 2B), PD + LD displayed the highest charge of the two microbiological parameters analysed ($1.4\text{E}+08 \text{ CFU g}^{-1}$ of MAC_{GMC} and $9.1\text{E}+04 \text{ CFU g}^{-1}$ of SFC_{GMC} , ca. two and three times higher compared to the average charges, respectively), followed by PD + SS ($8.7\text{E}+07 \text{ CFU g}^{-1}$ of MAC_{GMC} and $2.7\text{E}+02 \text{ CFU g}^{-1}$ of SFC_{GMC}).

Hydroponic system ^a	MAC_{GMC} (CFU g^{-1})	SFC_{GMC} (CFU g^{-1})
A. 1st and 2nd crop cycles		
AG + SS	$7.4\text{E}+05 \text{ c}$	$3.0\text{E}+03 \text{ c}$
AG + LD	$1.3\text{E}+06 \text{ b}$	$2.5\text{E}+03 \text{ c}$
SD + SS	$8.6\text{E}+05 \text{ c}$	$1.5\text{E}+03 \text{ c}$
SD + LD	$3.6\text{E}+06 \text{ a}$	$1.3\text{E}+05 \text{ a}$
S + SS	$4.0\text{E}+05 \text{ c}$	$8.0\text{E}+04 \text{ b}$
Average	$1.4\text{E}+06$	$4.3\text{E}+04$
B. 3rd crop cycle		
PM + SS	$4.5\text{E}+05 \text{ c}$	$7.7\text{E}+02 \text{ b}$
PM + LD	$3.9\text{E}+05 \text{ c}$	$8.0\text{E}+02 \text{ b}$
PD + SS	$8.7\text{E}+07 \text{ b}$	$2.7\text{E}+02 \text{ b}$
PD + LD	$1.4\text{E}+08 \text{ a}$	$9.1\text{E}+04 \text{ a}$
Average	$5.7\text{E}+07$	$2.3\text{E}+04$

Table 2. Microbiological quality of the growing media and nutrient solution combinations. Means followed by the same letter do not significantly differ at $P < 0.05$. AG = agriperlite; LD = liquid digestate; S = soil; SD = solid digestate; SS = standard solution; PM = peat moss; PD = pelletized digestate; MAC_{GMC} = mesophilic aerobic charge of the growing media and nutrient solution combinations; SFC_{GMC} = spore forming charge of the growing media and nutrient solution combinations. See text for details.

294

295 3.2. Baby leaf lettuce - agronomical and microbiological results

296 Innovative GM or NS obtained by the valorisation of by-products should be evaluated before being
 297 used for crops cultivation due to their possible phytotoxic or bio-stimulation effects, caused by their
 298 chemical and microbiological content. The results reported in Table S1 on germination assay,
 299 demonstrated that all GM, NS and their combinations showed values higher than 50% which might

300 be considered the phytotoxicity threshold reported by Zucconi et al. (1981). In particular, the
 301 combinations of water both using LD and SD proved the highest value of germination index (105%
 302 and 101%, respectively), while the lowest one was reported using PM (65%).

303 Growing media and NS performances were assessed on baby leaf lettuce in term of agronomical
 304 and microbiological parameters, in different experiments (three) and hydroponic systems (nine). In
 305 Table 3 are reported the production, nutrition status and microbiological ~~trait~~parameters recorded at
 306 the harvest time, regarding the first crop cycle using temperature at 24 ± 2 °C. Interesting statistical
 307 differences were observed for all the ~~trait~~parameters apart from shoot dry weight-height ratio and
 308 SPAD index. Focusing the attention on the most important ~~trait~~parameter such as shoot dry weight,
 309 the hydroponic systems SD + SS (0.85 g plot⁻¹) and AG + LD (0.82 g plot⁻¹) displayed the highest
 310 values (+42% and +37% compared to the general average, respectively). Moreover, the hydroponic
 311 system AG + LD recorded also the highest value of root (1.30 g plot⁻¹) and total dray weight (2.12 g
 312 plot⁻¹) (+86% and +56% compared to the general average, respectively). Finally, the hydroponic
 313 system AG + LD showed a drastic reduction of MAC_L (-76% respect to the average value of all
 314 others) and total absence of CC_L; similar microbiological results were recorded by the hydroponic
 315 system AG + SS, used as control in the present study. The hydroponic systems SD + SS and AG +
 316 SS showed the highest value of leaves height (+25 cm and +22 cm compared to the general average,
 317 respectively), S + SS one (used as another control), recorded the highest harvest index (+25%
 318 respect to the general average).

Hydroponic system ^a	H (cm)	SDW (g pot ⁻¹)	RDW (g pot ⁻¹)	TDW (g pot ⁻¹)	HI	SDW/H (g cm ⁻¹)	SPAD	MAC _L (UFC g ⁻¹)	CC _L (UFC g ⁻¹)
AG + SS	9.00 a	0.64 ab	0.87 ab	1.52 ab	0.43 bc	0.07 n.s.	11.33 n.s.	0 c	0 d
AG + LD	6.67 b	0.82 a	1.30 a	2.12 a	0.39 c	0.13 n.s.	13.80 n.s.	9.65E+01 c	0 d
SD + SS	9.17 a	0.85 a	0.83 ab	1.68 ab	0.53 ab	0.10 n.s.	13.03 n.s.	3.00E+02 b	8.50E+02 c

SD + LD	7.07 b	0.45 b c	0.37 bc	0.82 bc	0.55 ab	0.06 n.s.	13.93 n.s.	1.60E+03 a	6.10E+03 a
S + SS	4.83 c	0.22 c	0.12 c	0.34 c	0.64 a	0.04 n.s.	12.17 n.s.	5.00E+01 c	3.10E+03 b
Average	7.35	0.60	0.70	1.36	0.51	0.08	12.85	4.09E+02	2.01E+03

Table 3. Production, nutrition status and microbial charge of baby lettuce grown on different substrates and nutrient solutions in the first crop cycle (24 ± 2 °C). Means followed by the same letter do not significantly differ at $P < 0.05$; n.s. = not significantly different; AG = agriperlite; SS = standard solution; LD = liquid digestate; SD = solid digestate; S = soil; MAC_L = mesophilic aerobic charge of lettuce; CC_L = Coliform charge of lettuce; H = plant height; SDW = shoot dry weight; RDW = root dry weight; TDW = total dry weight; HI = harvest index; SPAD. See text for details.

In the second crop cycle an increase of three degrees Celsius during the baby leaf lettuce cultivation was investigated. In Table 4, are reported the production, nutrient status and microbiological charges of baby leaf lettuce grown at 27 ± 2 °C. Also, in the second crop cycle, interesting statistical differences were observed for all the ~~trait~~parameters recorded at the harvest time, apart from shoot dry weight-height ratio and HI. The hydroponic systems SD + SS and AG + LD recorded the highest shoot dry weight (0.74 g plot^{-1} and 0.72 g plot^{-1} , respectively) performing as well as in the first experiment. However, some interesting differences were highlighted. In the second crop cycle, the hydroponic system SD + SS showed the highest values of both root dry weight ($0.34 \text{ g plant}^{-1}$) and total dry weight ($1.08 \text{ g plant}^{-1}$) (+48% and +35% compared to the general average, respectively) and total absence of microbiological charge both for MAC_L and CC_L, and similar low microbiological charges were showed by AG + SS. Finally, the hydroponic system SD + SS recorded also the highest value of leaves height (+20% respect the general average), and the higher values of SPAD index (+12% compared to the general average), and similar indices of SPAD were recorded by AG + SS and AG + LD.

Hydroponic system ^a	H (cm)	SDW (g pot ⁻¹)	RDW (g pot ⁻¹)	TDW (g pot ⁻¹)	HI	SDW/H (g cm ⁻¹)	SPAD	MAC _L (UFC g ⁻¹)	CC _L (UFC g ⁻¹)
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AG + SS	9.67 b	0.54 ab	0.13 b	0.67 bc	0.82 n.s.	0.06 n.s.	12.90 a	0 c	1.10E+01 c
AG + LD	9.67 b	0.72 a	0.31 a	1.03 ab	0.71 n.s.	0.07 n.s.	13.77 a	3.50E+02 b	2.60E+01 b
SD + SS	11.17 a	0.74 a	0.34 a	1.08 a	0.69 n.s.	0.07 n.s.	13.20 a	0 c	0 d
SD + LD	10.00 b	0.53 ab	0.27 ab	0.80 abc	0.68 n.s.	0.05 n.s.	9.17 b	4.95E+03 a	4.00E+03 a
S + SS	5.83 c	0.31 b	0.13 b	0.43 c	0.66 n.s.	0.05 n.s.	9.83 b	4.00E+02 b	1.00E+01 cd
Average	9.27	0.57	0.23	0.80	0.71	0.06	11.77	1.14E+03	8.09E+02

Table 4. Production, nutrition status and microbial charge of baby lettuce grown on different substrates in the second crop cycle (27 ± 2 °C). Means followed by same letter do not significantly differ at $P < 0.05$; n.s. = not significantly different; AG = agriperlite; SS = standard solution; LD = liquid digestate; SD = solid digestate; S = soil; MAC_L = mesophilic aerobic charge of lettuce; CC_L = Coliform charge of lettuce; H = plant height; SDW = shoot dry weight; RDW = root dry weight; TDW = total dry weight; HI = harvest index; SPAD. See text for details.

The results of the third crop cycle, regarding the assessment of PM vs PD as growing media and liquid digestate and standard solution as nutrient solution, are reported in Table 5. The hydroponic system PD + SS, displayed the highest values of shoot, root and total dry weights, shoot dry weight-height ratio and SPAD index (+23, +53, +29, +88, 21% compared to the general average, respectively), and similar value of SPAD index was recorded by PM + SS (+9%). Moreover, PD + SS showed the lowest value of leaves height (-26% respect the general average) and the lower harvest index (-5% compared to the general average) and similar value of HI was reported by PD + LD (-6%).

Hydroponic system^a	H (cm)	SDW (g pot⁻¹)	RDW (g pot⁻¹)	TDW (g pot⁻¹)	HI	SDW/H (g cm⁻¹)	SPAD	MAC_L (UFC g⁻¹)	CC_L (UFC g⁻¹)
PM + SS	9.50 a	0.66 bc	0.10 b	0.76 c	0.87 a	0.07 c	15.95 a	7.00E+02 n.s.	1.20E+02 n.s.
PM + LD	8.66 b	0.54 c	0.08 b	0.62 c	0.86 a	0.06 c	11.43 b	6.00E+02 n.s.	5.00E+01 n.s.
PD + SS	5.60 d	0.85 a	0.26 a	1.11 a	0.77 b	0.15 a	17.63 a	3.00E+02 n.s.	3.50E+01 n.s.

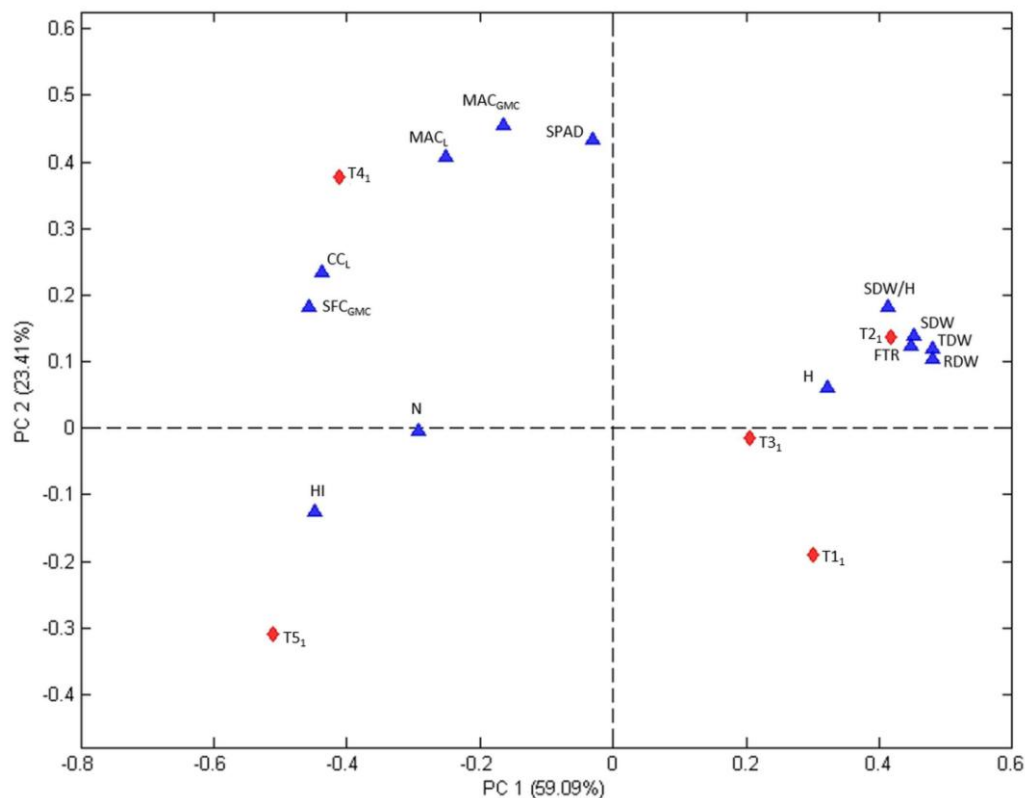
PD + LD	6.33 c	0.72 ab	0.22 a	0.94 b	0.76 b	0.11 b	13.33 b	8.00E+02 n.s.	1.00E+01 n.s.
Average	7.52	0.69	0.17	0.86	0.81	0.08	14.59	6.00E+02	5.38E+01

Table 5. Production, nutrition status and microbial charge of baby lettuce grown on different substrates in the third crop cycle (24 ± 2 °C). Means followed by same letter do not significantly differ at $P < 0.05$; n.s. = not significantly different; PM = peat moss; SS = standard solution; LD = liquid digestate; PD = pelletized digestate; MAC_L = mesophilic aerobic charge of lettuce; CC_L = Coliform charge of lettuce; H = plant height; SDW = shoot dry weight; RDW = root dry weight; TDW = total dry weight; HI = harvest index; SPAD. See text for details.

351

352 3.4 Relationships between recorded parameters and hydroponic systems

353 The correlations between data of the hydroponic systems variables measured on baby leaf lettuce
354 were studied by PCA analysis. Figures 1, 2 and 3 report ordination biplots of the PCA output
355 modelling for the three crop cycles investigated in the present work. For the first crop cycle, PC1
356 accounted for 59.09% of the variance, and PC2 accounted for 23.41%, and their sum explained
357 82.50% of total variance (Figure 1). The hydroponic systems T2₁ (AG + LD) and T3₁ (SD + SS)
358 were positively associated with the descriptive ~~trait~~parameters regarding biomass such as shoot,
359 root and total dry weight, leaves height, biomass fraction to root and shoot dry weight-height ratio
360 and negatively associated with baby leaf lettuce nitrate content and microbiological parameters.
361 While, the hydroponic system T4₁ (SD + LD) was closely associated with microbiological
362 parameters such as CC_L , MAC_{GMC} , MAC_L , SFC_{GMC} and SPAD index. Finally, hydroponic system
363 T5₁ (S + SS) was associated to HI.



364

365

366 **Figure 1.** Ordination biplot of principal component analysis of the first crop cycle (24 ± 2 °C). Labels in the graph
 367 represent the investigated parameters: CC_L = clostridium charge of baby leaf lettuce; SDW= shoot dry weight; RDW=
 368 root dry weight; TDW = total dry weight; H= plant height; HI = harvest index; SDW/H = shoot dry weight-height ratio;
 369 FTR = biomass fraction of dry weight to root; MAC_{GMC} = mesophilic aerobic charge of the growing media
 370 combinations; MA_{CL} = mesophilic aerobic charge of baby leaf lettuce; SFC_{GMC} = spore-forming charge of the growing
 371 media combinations; SPAD; N = baby leaf lettuce nitrate content; T1-T5 = the different hydroponic systems: T1 = AG +
 372 SS; T2 = AG + LD; T3 = SD + SS; T4 = SD + LD; T5 = S + SS. AG = agriperlite; LD = liquid digestate; S = soil; SD =
 373 solid digestate; SS = standard solution. Number 1 following the hydroponic systems investigated indicate the
 374 corresponding crop cycle (the first one).

375

376 Regarding the second crop cycle investigated, PC1 accounted for 47.54% of the variance, and PC2
 377 accounted for 37.00%, and their sum explained 84.54% of total variance (Figure 2). The hydroponic
 378 systems T2₂ (AG + LD) and T3₂ (SD + SS) were closely associated with the descriptive
 379 traitparameters regarding biomass (shoot, root and total dry weight, leaves height, shoot dry weight-
 380 height ratio and SPAD index); on the contrary, it was negatively correlated with baby leaf lettuce
 381 nitrate content and microbiological parameters, as well as showed in the PCA profile of the first
 382 experiment, except for SPAD index and biomass fraction to root. Also, the hydroponic system T4₂

(SD +LD) confirmed its association with microbiological parameters such as CC_L , MAC_{GMC} , MA_{CL} and SFC_{GMC} as highlighted in the PCA result of the first experiment. Finally, hydroponic system $T1_2$ (AG + SS) was well correlated to HI.

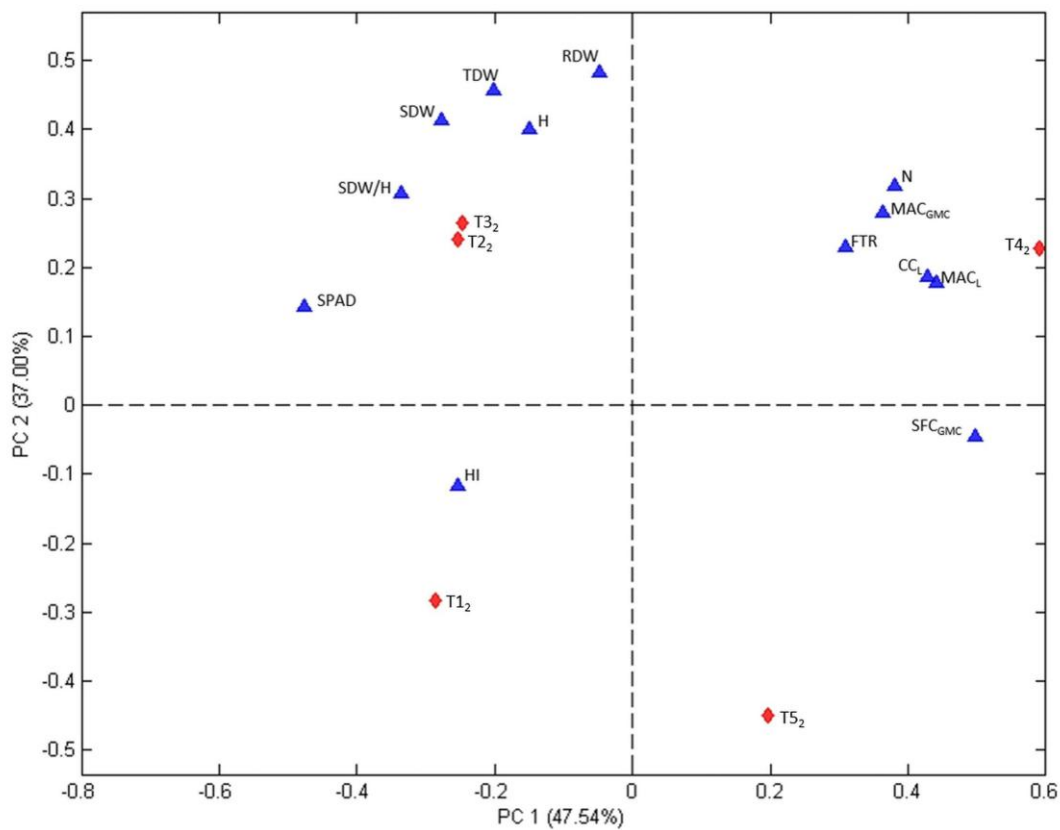
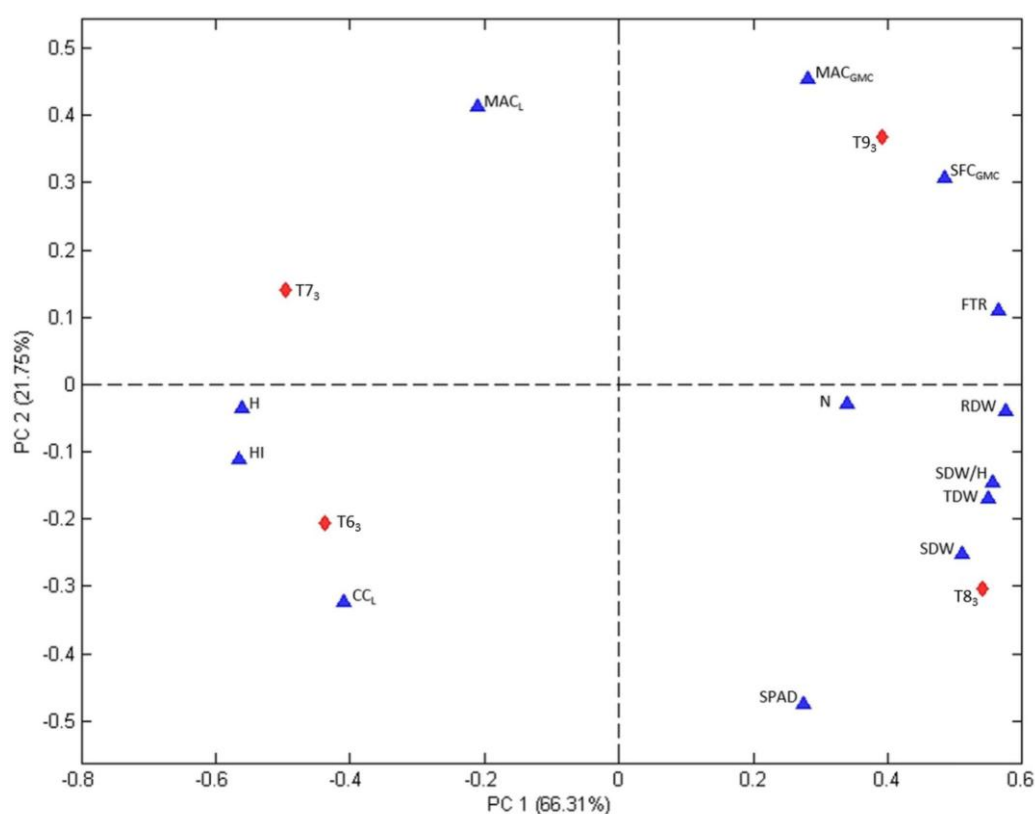


Figure 2. Ordination biplot of principal component analysis of the second crop cycle (27 ± 2 °C). Labels in the graph represent the investigated parameters: CC_L = clostridium charge of baby leaf lettuce; SDW= shoot dry weight; RDW= root dry weight; TDW = total dry weight; H= plant height; HI = harvest index; SDW/H = shoot dry weight-height ratio; FTR = biomass fraction of dry weight to root; MAC_{GMC} = mesophilic aerobic charge of the growing media combinations; MA_{CL} = mesophilic aerobic charge of baby leaf lettuce; SFC_{GMC} = spore-forming charge of the growing media combinations; SPAD; N = baby leaf lettuce nitrate content; T1-T5 = the different hydroponic systems: T1 = AG + SS; T2 = AG + LD; T3 = SD + SS; T4 = SD + LD; T5 = S + SS. AG = agriperlite; LD = liquid digestate; S = soil; SD = solid digestate; SS = standard solution. Number 2 following the hydroponic systems investigated indicate the corresponding crop cycle (the second one).

Summarizing across the two first crop cycles investigated, SDW, RDW, TDW, H and SDW/H were the ~~trait~~parameters most consistently associated to the hydroponic systems AG + LD and SD + SS. In the third crop cycle, PM vs PD were investigated as growing media. As reported in Figure 3, PC1 accounted for 66.31% of the variance, and PC2 accounted for 21.75%, and their sum explained

391 88.06% of the total variance. The hydroponic system T8₃ (PD + SS) was high correlated and mainly
 392 influenced by descriptive ~~trait~~ parameters regarding biomass (shoot and total dry weight, shoot dry
 393 weight-height ratio, and SPAD index), while the hydroponic system T9₃ (PD + LD) was closely
 394 associated with the microbiological parameters relates to the initial charges contained in the
 395 growing media and nutrient solution investigated (MAC_{GMC}, and SFC_{GMC}). Finally, the hydroponic
 396 system T6₃ (PM + SS) was correlated to HI and CC_L.



397

398

Figure 3. Ordination biplot of principal component analysis of the third crop cycle (24 ± 2 °C). Labels in the graph represent the investigated parameters: CC_L = clostridium charge of baby leaf lettuce; SDW= shoot dry weight; RDW= root dry weight; TDW = total dry weight; H= plant height; HI = harvest index; SDW/H = shoot dry weight-height ratio; FTR = biomass fraction of dry weight to root; MAC_{GMC} = mesophilic aerobic charge of the growing media combinations; MAC_L = mesophilic aerobic charge of baby leaf lettuce; SFC_{GMC} = spore-forming charge of the growing media combinations; SPAD; N = baby leaf lettuce nitrate content; T6-T9 = the different hydroponic systems: T6 = PM + SS; T7 = PM + LD; T8 = PD + SS; T9 = PD + LD; LD = liquid digestate; PM = peat moss; PD = pelletized digestate; SD = solid digestate; SS = standard solution. Number 3 following the hydroponic systems investigated indicate the corresponding crop cycle (the third one).

399

400 4. Discussion

401 In literature there are works whose main aim were testing the use of digestate as fertilizer, for the
402 cultivation in open field (Lukehurst et al., 2010; Makádi et al., 2012). Despite the fact that some
403 agronomic studies on aerobic and anaerobic digestion have been performed (Goddek et al., 2016;
404 Stoknes et al., 2016), the impact of both SD and LD on plant growth is not completely clear. In
405 addition, as far as the present state of art on this topic, the information regarding the use of digestate
406 as fertilizer in greenhouse is limited, especially for soilless systems (Liedl et al., 2006). Hence, the
407 present study aimed to analyse the effects of digestates on yield and other agronomical and
408 microbiological as alternative and sustainable growing media and nutrient solution for the
409 cultivation of baby leaf lettuce using hydroponics.

410 Baby leaf vegetables are fresh foods that are frequently linked with food borne outbreaks (Nicola et
411 al., 2009). In fact, contaminations with pathogenic microorganisms might have occurred during
412 crop cycle due to the contact with soil and irrigation water (Tournas, 2005). In order to adopt
413 strategies that could minimize the risk of microbiological contamination within agricultural system,
414 it is important to understand the charge of pathogens, in growing media and soils and how their
415 might influence the contamination (Nicola et al., 2009). In the present study, SD and LD reported
416 the highest charge of MAC and SFC, respectively (Table 1). Moreover, the hydroponic system SD +
417 LD and PD + LD highlighted the highest charge of MAC_{GMC} and SFC_{GMC} (Table 2). The presence
418 of *Clostridium spp.* bacteria observed in the digestate was already reported in earlier studies (Bagge
419 et al., 2005, Bonetta et al., 2011). In general, anaerobic digestion does not reduce *Clostridium spp.*
420 content (Bagge et al., 2005). The genus *Clostridium* survived in the anaerobic digestion process
421 (Schnurer and Jarvis, 2009) because only vegetative cells are susceptible to temperatures above 50
422 °C, while the elimination of spores requires further and more intense heat-treatments
423 (Watcharasukarn et al., 2009). Bagge et al. (2005) reported that pathogen regrowth during storage

424 was probably due to non-hygienic conditions of the storage tanks, as showed for pelleted digested in
 425 the present work versus the data reported by Pulvirenti et al. (2015).
 426 Soil growing media might influence both the germination and the emergence of seedlings. In the
 427 present study, the germination assays indicated that there were no phytotoxicity issues in the
 428 analysed growing media, nutrient solutions and their combinations. In fact, they showed values of
 429 the germination index greater than 50% (Table S1), which may be considered as a threshold value
 430 for phytotoxicity (Zucconi et al. 1981). In particular, GI% proved greater values for H₂O + LD and
 431 H₂O + SD, thus they showed a biostimulant effect might due to digestate content. In fact, Yu et al.
 432 (1995) confirmed that the germination power and percentage should increase in seeds previously
 433 soaked in LD. Moreover, Gell et al. (2011) and Sánchez et al. (2008) obtained similar results
 434 evaluating the digestate phytotoxicity on lettuce, radish, wheat, and garden cress. On the other hand,
 435 the combined use of digestate as GM and NS slightly decreased the germination index values of the
 436 other investigated treatments, probably due their pH values. Hence, although depending on species
 437 growth and yield adaptation, this constitutes a limit for the use of digestate without pH correction as
 438 already reported by Endo et al. (2016). Moreover the phytotoxicity of digestate could be related to
 439 the presence of $\text{NH}_4^+ - \text{NNH}^{4+} - \text{N}$ and organic acids (Möller and Müller, 2012).
 440 As far as the agronomical investigated ~~trait~~parameters, our results were in agreement with
 441 Vimolmangkang et al. (2010) who showed how “deep flow” technique increased mint growth. Baby
 442 leaf total dry weight was improved by ca. two-fold using hydroponic systems respect to soil (Table
 443 3 and 4). In addition, taking into account the most important ~~trait~~parameters such as shoot dry
 444 weight in the first and the second crop cycles (Table 1 and 2), the hydroponic systems AG + LD and
 445 SD + SS reported the highest values, which were probably due to the presence in SD of unknown
 446 compounds/molecules either acting as, or mimicing plant growth promoters. In fact, previous
 447 studies have shown that digestates contain phytohormones – above all, gibberellins, indole acetic
 448 acid, auxin-like and auxin-active molecules – dissolved in the organic matter, and other bioactive

449 compounds that have the potential to promote plant growth and to increase the tolerance to biotic
450 and abiotic stresses (Liu et al., 2009; Scaglia et al., 2017; Yu et al., 2010).

451 Comparing the two investigated temperatures, RDW and TDW showed higher values, while HI
452 lower, at 24 °C rather than 27 °C. Moreover, in the present study soilless cropping reduces harvest
453 index compared to soil. However, this reduction was ascribed to a higher increase of root growth
454 than of shoot growth in hydroponics, as already reported by Olympios (1999). This growth
455 acceleration, especially in root, was due to a more and constant availability of nutrients as showed
456 in processing tomato (Ronga et al., 2017). Moreover, several studies compared the crop cultivation
457 in soil vs soilless systems highlighting that soilless reduces the crop cycle and increase crop yield
458 (Fontana and Nicola, 2009; Incrocci et al., 2001), and the latter was shown in the present study.

459 Regarding the nutritional status, SPAD index values recorded in the second and third experiments
460 were in accordance with Chrysargyris et al. (2017) who showed that nitrogen levels affected plant
461 growth and chlorophyll. On the other hand, the same trend was not recorded in the first experiment
462 probably due to the low temperature that did not allow the same availability of the nutrients,
463 bioactive compounds and microorganism contained in the solid digestate. However, further studies
464 are needed to confirm these hypotheses.

465 Food safety management in the fresh-cut chain is expected before processing, thus the food safety
466 risks depend on genotypes, management, environment and their interactions (Kirezieva et al.,
467 2013). The microbial contamination of lettuce irrigated using the furrow system was much lower
468 than lettuce irrigated using sprinklers (Fonseca, 2006). Moreover, processing operations might
469 contaminate fresh vegetables if the edible portions were in direct contact with water or soil
470 containing pathogens (Solomon et al., 2003). The microbiological analysis of the baby leaf lettuce
471 demonstrates low level of aerobic mesophilic contamination as shown in the Tables 3 to 5. In fact,
472 no sample had a level above $5.0E+05 \cdot CFU \text{ g}^{-1}$ of product. This is the safety threshold for selling
473 fresh vegetables (HPA, 2009). The *Coliform* analysis showed a very low charge under the selling

threshold ($1.0\text{E}+03 \text{ CFU g}^{-1}$), except for the theses S + SS, SD + SS, SD + LD and PM + SS that have a *Coliform* charge higher than the threshold and reach a maximum of $1.0\text{E}+03 \text{ CFU g}^{-1}$ with the thesis SD + LD (Tables 3 and 4) (HPA, 2009). In general, vegetables cropped in the open field reach a total bacterial count of $1.0\text{E}+06$ to $1.0\text{E}+09 \text{ CFU g}^{-1}$, which might be reduced by 2-3 log CFU g^{-1} after washing practices (Nicola and Fontana, 2014; Selma et al., 2012) confirming the results obtained in the present study, where the leaves were microbiological analysed without washing. However, in baby leaf vegetables washing operations are crucial to make the product ready-to-eat and will able to reduce the microbiological charges recorded in the present study. Finally, baby leaf vegetables should be clean, free of soil residue, insects, metals and weeds.

Analysing the relationships between recorded parameters and hydroponic systems, from the PCA analysis of the first and second crop cycle emerged that the hydroponic systems T2 and T3 are associated with SDW, TDW, RDW and FTR, while the T4 is related with CC_L , MAC_L and MAC_{GMC} . Regarding to the PCA analysis of the third crop cycle, the T8 was related with SDW, TDW and SPAD and the T9 is connected with MAC_{GMC} and SFC_{GMC} . Finally, the T6 was associated with CC_L , HI and H. In general, the digestates used as growing media (solid and pelleted) performed better using standard solution as nutrient solution in all three experiments, probably due to a better balance of organic and mineral nutrient availability. However, further researches are needed to corroborate this hypothesis. In vegetables, quality ~~trait~~parameters such as firmness, dry matter percentage and soluble sugar content are negatively correlated with nitrogen content. An excess of nitrogen availability might increase crop susceptibility to biotic and biotic stress, however, neither were recorded in the present study (data not shown). Moreover, the hydroponic combination that performed better in each experiment (T2, T3 and T8) were negatively correlated with the nitrate content in baby leaf samples (Figure 1-3). In the EU, there is a specific regulation (EU Reg. 1258/2011, amending EU Reg. 1881/2006 that amended EU-Reg. N. 563/2002) that sets the threshold levels of nitrate content (below $2500 \text{ mg kg}^{-1} \text{ f.w.}$) in the edible

499 part of vegetables such as lettuce (*Lactuca sativa* L.), and in the present study all samples were
 500 below the threshold ranging between 338 and 1640 mg kg⁻¹ f.w. ([Table S1](#) ~~data not shown~~).
 501 The most striking differences between the agriperlite and the digestate were recorded for the
 502 combination of AG + LD and SD + SS that improved the shoot dry weight of baby leaf lettuce both
 503 in the first and in the second experiment (Figure 4), while the combination of SD + LD did not
 504 perform as well.



505
 506 **Figure 4.** The five representative pots of baby leaf lettuce cultivated in the first and second cycle. AG + SS = agriperlite
 507 + standard solution; AG + LD = agriperlite + liquid digestate; SD + SS = solid digestate + standard solution; SD + LD =
 508 solid digestate + liquid digestate; S + SS = soil + standard solution.

509
 510 The similar trend was highlighted during the third experiment where PD + SS and PD + LD
 511 performed better than the other investigated hydroponic systems (Figure 5).



512
 513 **Figure 5.** The four representative pots of baby leaf lettuce cultivated in the third cycle. PM + SS = peat moss + standard
 514 solution; PM + LD = peat moss + liquid digestate; PD + SS = pelleted digestate + standard solution; PD + LD =
 515 pelleted digestate + liquid digestate.

516
 517 The lower performance showed by hydroponic system SD + LD and PD + LD could be caused by
 518 inorganic nitrogen content in the digestate was provided as a high concentration of NH₄⁺ and high
 519 pH value. In fact, Endo et al. (2016) reported inhibition when cucumber seedlings were grown
 520 hydroponically with digestate as nutrient solution. [However, the feasibility of nitrification could be](#)

521 tested also on digestates. In fact, Cáceres et al. (2015) using the nitrified leachates formed during
522 composting of cattle and pig manure, as liquid fertilizer, obtained similar lettuce productivities in
523 comparison to the standard nutritive solution.

524 Finally, pellet digestate could be an interesting alternative growing media. In fact, digestate
525 transport from farms located farther than 20 km from the AD plants is convenient in terms of
526 associated costs and carbon footprint; therefore, this alternative way that reduces the overall
527 environmental impacts of AD plants might improve the economic value of digestate and the
528 agricultural sustainability.

529 **5. Conclusions**

530 ~~The present study underlined how digestates might improve the sustainability of baby leaf lettuce in~~
531 ~~hydroponics. Nowadays, there are just a few studies about the use of digestate in soilless systems;~~
532 ~~so, the availability of data to compare the results obtained is scarce.~~ Considerable effort has been
533 made in the search of improved sustainability of hydroponics, and microbiological control for fresh-
534 cut vegetables. Nowadays, there are just a few studies about the use of digestate in soilless systems;
535 so, the availability of data to compare the results obtained is scarce. The present study underlined
536 how digestates might improve the sustainability of baby leaf lettuce in hydroponics. The
537 combination of agriperlite + liquid digestate, solid digestate + standard solution and pelleted
538 digestate + standard solution recorded higher values of root, shoot and total dry weights and SPAD,
539 compared to the average value of the all assessed treatments, in the all investigated experiments.

540 This study highlighted the possible use of solid and liquid digestates as growing media ~~and~~
541 nutrient solution, respectively to grow baby leaf lettuce with high yield and low microbiological
542 contaminations. ~~Solid and liquid~~ Digestates for hydroponic lettuce cultivation show a great
543 potential in the future of hydroponic greenhouse due to its low cost, environment sustainability, and
544 interesting agronomical and microbiological ~~trait~~ parameters. However, further studies are needed to

improve the combined use of solid and liquid digestates, despite this, the baby leaf lettuce produced in this way showed a great potential for the scale-up.

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Medium and nutrient solutions ^a	MAC (CFU g ⁻¹)	SFC (CFU g ⁻¹)
SD	8.3E+06 a	5.5E+05 b
PM	6.4E+05 b	0 c
S	5.2E+05 c	1.3E+04 c
AG	5.2E+03 d	1.1E+03 c
LD	7.6E+03 d	7.3E+05 a
PD	1.5E+03 d	1.1E+03 c
SS	0 d	0 c
VE	1.1E+03 d	2.3E+02 c
Average	1.2E+06	1.6E+05

Table 1. Microbiological quality of the growing media and nutrient solutions. Means followed by the same letter do not significantly differ at P<0.05. AG = agriperlite; LD = liquid digestate; PD = pelletized digestate; PM = peat moss; S = soil; SD = solid digestate; SS = standard solution; VE = vermiculite; MAC = mesophilic aerobic charge; SFC = spore forming charge. See text for details.

Hydroponic system ^a	MAC _{GMC} (CFU g ⁻¹)	SFC _{GMC} (CFU g ⁻¹)
A. 1st and 2nd crop cycles		
AG + SS	7.4E+05 c	3.0E+03 c
AG + LD	1.3E+06 b	2.5E+03 c
SD + SS	8.6+05 c	1.5E+03 c
SD + LD	3.6E+06 a	1.3E+05 a
S + SS	4.0E+05 c	8.0E+04 b
Average	1.4E+06	4.3E+04
B. 3rd crop cycle		
PM + SS	4.5E+05 c	7.7E+02 b
PM + LD	3.9E+05 c	8.0E+02 b
PD + SS	8.7E+07 b	2.7E+02 b
PD + LD	1.4E+08 a	9.1E+04 a
Average	5.7E+07	2.3E+04

Table 2. Microbiological quality of the growing media and nutrient solution combinations. Means followed by the same letter do not significantly differ at P<0.05. AG = agriperlite; LD = liquid digestate; S = soil; SD = solid digestate; SS = standard solution; PM = peat moss; PD = pelletized digestate; MAC_{GMC} = mesophilic aerobic charge of the growing media and nutrient solution combinations; SFC_{GMC} = spore forming charge of the growing media and nutrient solution combinations. See text for details.

Hydroponic system ^a	H (cm)	SDW (g pot ⁻¹)	RDW (g pot ⁻¹)	TDW (g pot ⁻¹)	HI	SDW/H (g cm ⁻¹)	SPAD	MAC _L (UFC g ⁻¹)	CC _L (UFC g ⁻¹)
AG + SS	9.00 a	0.64 ab	0.87 ab	1.52 ab	0.43 bc	0.07 n.s.	11.33 n.s.	0 c	0 d
AG + LD	6.67 b	0.82 a	1.30 a	2.12 a	0.39 c	0.13 n.s.	13.80 n.s.	9.65E+01 c	0 d
SD + SS	9.17 a	0.85 a	0.83 ab	1.68 ab	0.53 ab	0.10 n.s.	13.03 n.s.	3.00E+02 b	8.50E+02 c
SD + LD	7.07 b	0.45 b c	0.37 bc	0.82 bc	0.55 ab	0.06 n.s.	13.93 n.s.	1.60E+03 a	6.10E+03 a
S + SS	4.83 c	0.22 c	0.12 c	0.34 c	0.64 a	0.04 n.s.	12.17 n.s.	5.00E+01 c	3.10E+03 b
Average	7.35	0.60	0.70	1.36	0.51	0.08	12.85	4.09E+02	2.01E+03

Table 3. Production, nutrition status and microbial charge of baby lettuce grown on different substrates and nutrient solutions in the first crop cycle (24 ± 2 °C). Means followed by the same letter do not significantly differ at $P < 0.05$; n.s = not significantly different; AG = agriperlite; SS = standard solution; LD = liquid digestate; SD = solid digestate; S = soil; MAC_L = mesophilic aerobic charge of lettuce; CC_L = Coliform charge of lettuce; H = plant height; SDW = shoot dry weight; RDW = root dry weight; TDW = total dry weight; HI = harvest index; SPAD. See text for details.

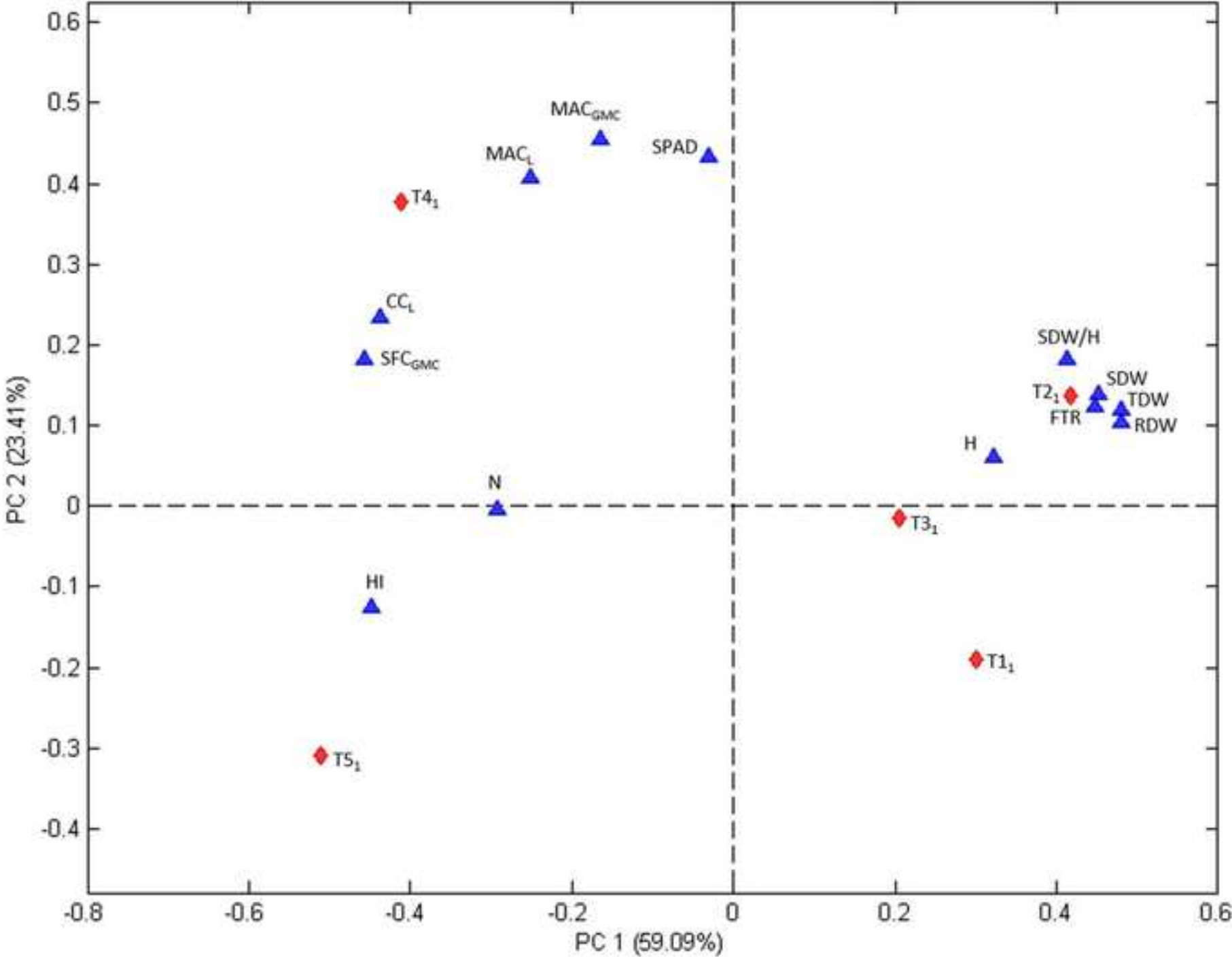
Hydroponic system ^a	H (cm)	SDW (g pot ⁻¹)	RDW (g pot ⁻¹)	TDW (g pot ⁻¹)	HI	SDW/H (g cm ⁻¹)	SPAD	MAC _L (UFC g ⁻¹)	CC _L (UFC g ⁻¹)
AG + SS	9.67 b	0.54 ab	0.13 b	0.67 bc	0.82 n.s.	0.06 n.s.	12.90 a	0 c	1.10E+01 c
AG + LD	9.67 b	0.72 a	0.31 a	1.03 ab	0.71 n.s.	0.07 n.s.	13.77 a	3.50E+02 b	2.60E+01 b
SD + SS	11.17 a	0.74 a	0.34 a	1.08 a	0.69 n.s.	0.07 n.s.	13.20 a	0 c	0 d
SD + LD	10.00 b	0.53 ab	0.27 ab	0.80 abc	0.68 n.s.	0.05 n.s.	9.17 b	4.95E+03 a	4.00E+03 a
S + SS	5.83 c	0.31 b	0.13 b	0.43 c	0.66 n.s.	0.05 n.s.	9.83 b	4.00E+02 b	1.00E+01 cd
Average	9.27	0.57	0.23	0.80	0.71	0.06	11.77	1.14E+03	8.09E+02

Table 4. Production, nutrition status and microbial charge of baby lettuce grown on different substrates in the second crop cycle (27 ± 2 °C). Means followed by same letter do not significantly differ at $P < 0.05$; n.s. = not significantly different; AG = agriperlite; SS = standard solution; LD = liquid digestate; SD = solid digestate; S = soil; MAC_L = mesophilic aerobic charge of lettuce; CC_L = Coliform charge of lettuce; H = plant height; SDW = shoot dry weight; RDW = root dry weight; TDW = total dry weight; HI = harvest index; SPAD. See text for details.

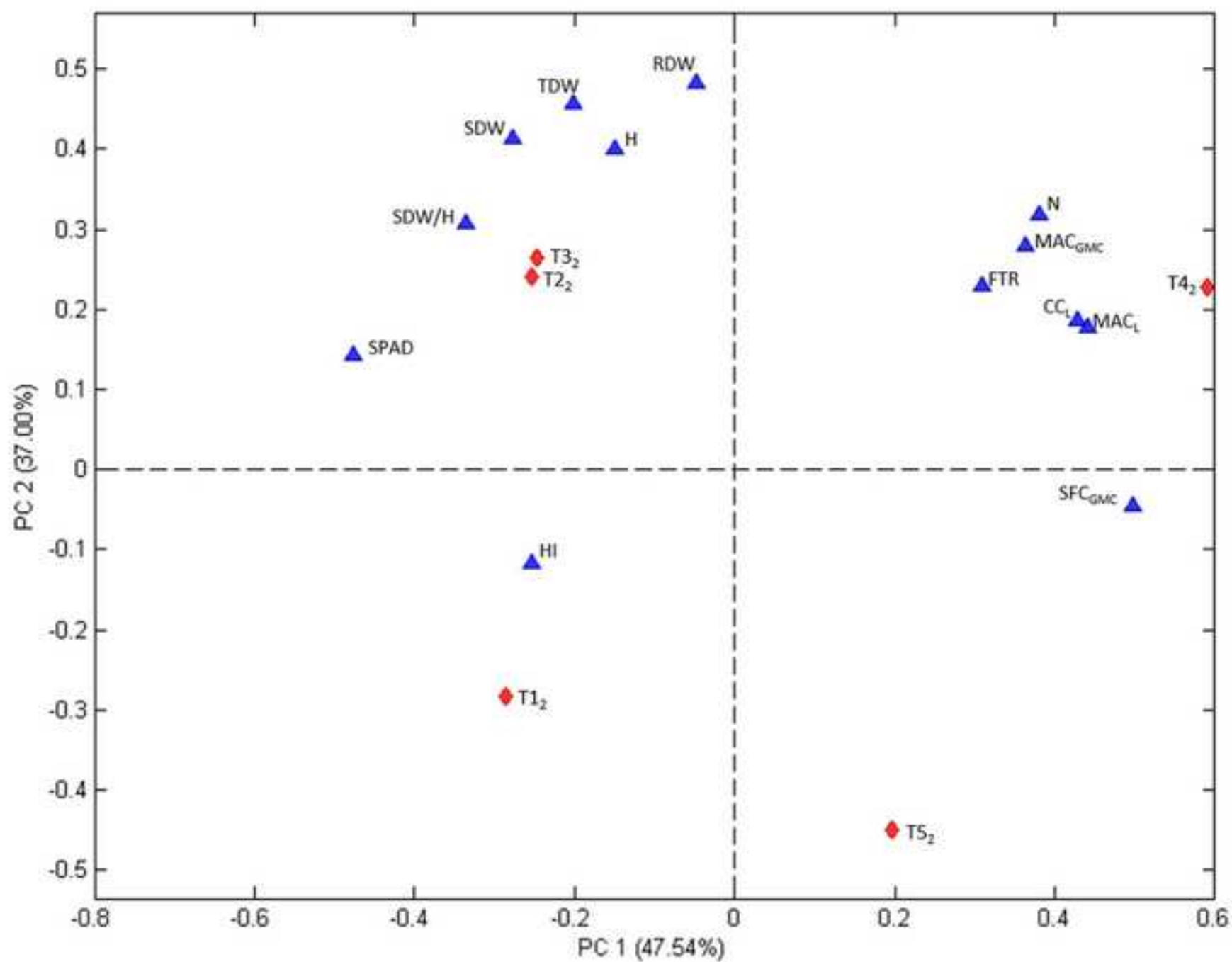
Hydroponic system ^a	H (cm)	SDW (g pot ⁻¹)	RDW (g pot ⁻¹)	TDW (g pot ⁻¹)	HI	SDW/H (g cm ⁻¹)	SPAD	MAC _L (UFC g ⁻¹)	CC _L (UFC g ⁻¹)
PM + SS	9.50 a	0.66 bc	0.10 b	0.76 c	0.87 a	0.07 c	15.95 a	7.00E+02 n.s.	1.20E+02 n.s.
PM + LD	8.66 b	0.54 c	0.08 b	0.62 c	0.86 a	0.06 c	11.43 b	6.00E+02 n.s.	5.00E+01 n.s.
PD + SS	5.60 d	0.85 a	0.26 a	1.11 a	0.77 b	0.15 a	17.63 a	3.00E+02 n.s.	3.50E+01 n.s.
PD + LD	6.33 c	0.72 ab	0.22 a	0.94 b	0.76 b	0.11 b	13.33 b	8.00E+02 n.s.	1.00E+01 n.s.
Average	7.52	0.69	0.17	0.86	0.81	0.08	14.59	6.00E+02	5.38E+01

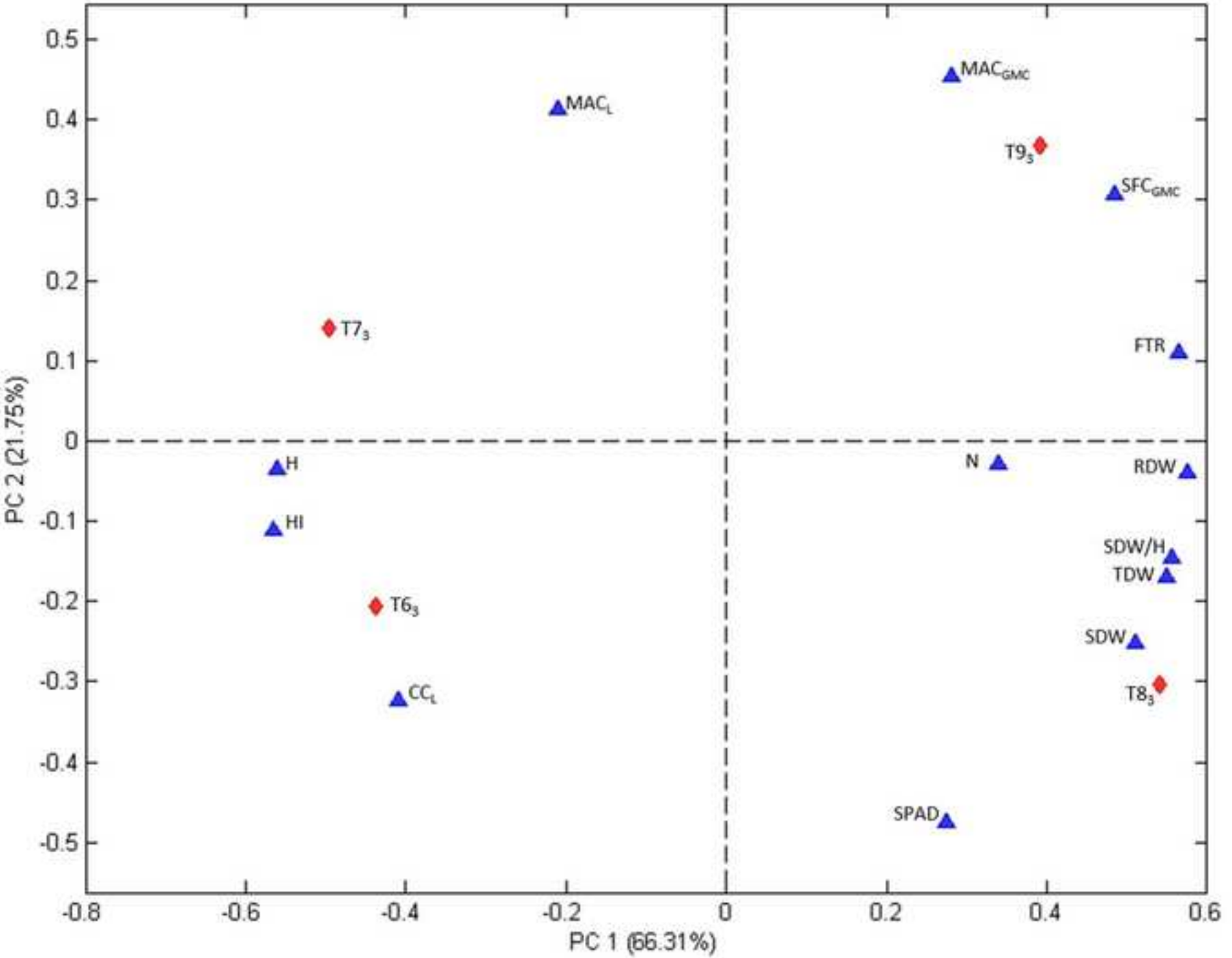
Table 5. Production, nutrition status and microbial charge of baby lettuce grown on different substrates in the third crop cycle (24 ± 2 °C). Means followed by same letter do not significantly differ at $P < 0.05$; n.s. = not significantly different; PM = peat moss; SS = standard solution; LD = liquid digestate; PD = pelletized digestate; MAC_L = mesophilic aerobic charge of lettuce; CC_L = Coliform charge of lettuce; H = plant height; SDW = shoot dry weight; RDW = root dry weight; TDW = total dry weight; HI = harvest index; SPAD. See text for details.

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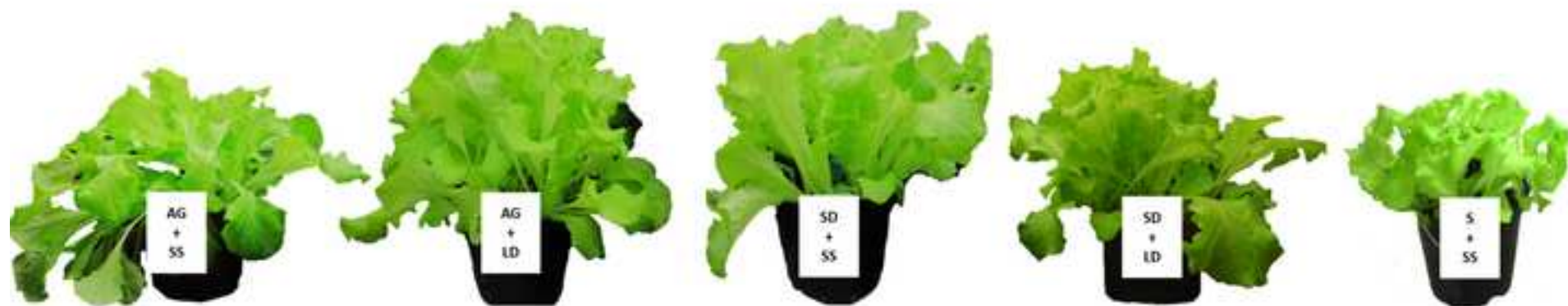
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Figure 1. Ordination biplot of principal component analysis of the first crop cycle (24 ± 2 °C). Labels in the graph represent the investigated parameters: CC_L = clostridium charge of baby leaf lettuce; SDW= shoot dry weight; RDW= root dry weight; TDW = total dry weight; H= plant height; HI = harvest index; SDW/H = shoot dry weight-height ratio; FTR = biomass fraction of dry weight to root; MAC_{GMC} = mesophilic aerobic charge of the growing media combinations; MA_{CL} = mesophilic aerobic charge of baby leaf lettuce; SFC_{GMC} = spore-forming charge of the growing media combinations; SPAD; N = baby leaf lettuce nitrate content; T1-T5 = the different hydroponic systems: T1 = AG + SS; T2 = AG + LD; T3 = SD + SS; T4 = SD + LD; T5 = S+SS. AG = agriperlite; LD = liquid digestate; S = soil; SD = solid digestate; SS = standard solution. Number 1 following the hydroponic systems investigated indicate the corresponding crop cycle (the first one).

Figure 2. Ordination biplot of principal component analysis of the second crop cycle (27 ± 2 °C). Labels in the graph represent the investigated parameters: CC_L = clostridium charge of baby leaf lettuce; SDW= shoot dry weight; RDW= root dry weight; TDW = total dry weight; H= plant height; HI = harvest index; SDW/H = shoot dry weight-height ratio; FTR = biomass fraction of dry weight to root; MAC_{GMC} = mesophilic aerobic charge of the growing media combinations; MA_{CL} = mesophilic aerobic charge of baby leaf lettuce; SFC_{GMC} = spore-forming charge of the growing media combinations; SPAD; N = baby leaf lettuce nitrate content; T1-T5 = the different hydroponic systems: T1 = AG + SS; T2 = AG + LD; T3 = SD + SS; T4 = SD + LD; T5 = S + SS. AG = agriperlite; LD = liquid digestate; S = soil; SD = solid digestate; SS = standard solution. Number 2 following the hydroponic systems investigated indicate the corresponding crop cycle (the second one).

Figure 3. Ordination biplot of principal component analysis of the third crop cycle (24 ± 2 °C). Labels in the graph represent the investigated parameters: CC_L = clostridium charge of baby leaf lettuce; SDW= shoot dry weight; RDW= root dry weight; TDW = total dry weight; H= plant height; HI = harvest index; SDW/H = shoot dry weight-height ratio; FTR = biomass fraction of dry weight

to root; MAC_{GMC} = mesophilic aerobic charge of the growing media combinations; MA_{CL} = mesophilic aerobic charge of baby leaf lettuce; SFC_{GMC} = spore-forming charge of the growing media combinations; SPAD; N = baby leaf lettuce nitrate content; T6-T9 = the different hydroponic systems: T6 = PM + SS; T7 = PM + LD; T8 = PD + SS; T9 = PD + LD; LD = liquid digestate; PM = peat moss; PD = pelletized digestate; SD = solid digestate; SS = standard solution. Number 3 following the hydroponic systems investigated indicate the corresponding crop cycle (the third one).

Figure 4. The five representative pots of baby leaf lettuce cultivated in the first and second cycles. AG + SS = agriperlite + standard solution; AG + LD = agriperlite + liquid digestate; SD + SS = solid digestate + standard solution; SD + LD = solid digestate + liquid digestate; S + SS = soil + standard solution.

Figure 5. The four representative pots of baby leaf lettuce cultivated in the third cycle. PM + SS = peat moss digestate + standard solution; PM + LD = peat moss + liquid digestate; PD + SS = pelleted digestate + standard solution; PD + LD = pelleted digestate + liquid digestate.

Supplementary Material

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