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Microbiological investigation of *Raphanus sativus* L. grown hydroponically in nutrient solutions contaminated with spoilage and pathogenic bacteria

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ABSTRACT

The survival of eight undesired (spoilage/pathogenic) food related bacteria (*Citrobacter freundii* PSS60, *Enterobacter* spp. PSS11, *Escherichia coli* PSS2, *Klebsiella oxytoca* PSS82, *Serratia grimesii* PSS72, *Pseudomonas putida* PSS21, *Stenotrophomonas maltophilia* PSS52 and *Listeria monocytogenes* ATCC 19114[T]) was investigated in mineral nutrient solution (MNS) during the crop cycle of radishes (*Raphanus sativus* L.) cultivated in hydroponics in a greenhouse. MNSs were microbiologically analyzed weekly by plate count. The evolution of the pure cultures was also evaluated in sterile MNS in test tubes. The inoculated trials contained an initial total mesophilic count (TMC) ranging between 6.69 and 7.78 Log CFU/mL, while non-sterile and sterile control trials showed levels of 4.39 and 0.97 Log CFU/mL, respectively. In general, all inoculated trials showed similar levels of TMC in MNS during the experimentation, even though the levels of the inoculated bacteria decreased. The presence of the inoculums was ascertained by randomly amplified polymorphic DNA (RAPD) analysis applied on the isolates collected at 7-day intervals. At harvest, MNSs were also analyzed by denaturing gradient gel electrophoresis (DGGE). The last analysis, except *P. putida* PSS21 in the corresponding trial, did not detect the other bacteria, but confirmed that pseudomonads were present in un-inoculated MNSs. Despite the high counts detected (6.44 and 7.24 CFU/g), only *C. freundii* PSS60, *Enterobacter* spp. PSS11 and *K. oxytoca* PSS82 were detected in radishes in a living form, suggesting their internalization.

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1. Introduction

Consumers are becoming more and more shrewd concerning alimentation; thus, their attention to the damages of unhealthy foods has determined an increase in the request for vegetable products in the last years. However, in many developed countries the consumption of vegetables is lower than the recommended dietary guidelines and an increase in the consumption of vegetables and fruits is strongly advised. Nevertheless, often consumers' dissatisfaction with produce quality (appearance, flavour, texture, perishability and hygienic safety) may limit their consumption (O'Beirne, 2007).

Minimally processed vegetables are creating an increase of requests for fresh produce due to their convenience of use and attractive appearance and flavour, but their benefits are offset by the rapid deterioration/short shelf-life of the products in the marketplace and the potential health hazards associated with microbial growth. Although it is somehow difficult to determine the exact route of contamination, the environment and the agricultural practices from planting through harvesting are of particular concern (Buck et al., 2003). At the pre-harvest stage, the crop management might limit or eliminate the risks of pathogenic microbial transference from soil to vegetables (Settanni et al., 2012).

Many factors may determine the microbial contamination also during the post-harvest manipulation (Martínez-Sánchez et al., 2006); the higher the concentrations of microorganisms the more intense the washing treatment of vegetables before consumption in the fresh-cut form needs to be. For vegetables whose edible parts grow underground, soil plays a defining role on the hygienic characteristics of the products. Soil may host several microorganisms (van Elsas et al., 2007), including bacterial species that are pathogenic for humans, such as members of the Enterobacteriaceae family (Forssten, 2009), *Listeria monocytogenes* (Welshimer and Donker-Voet, 1971), or *Stenotrophomonas maltophilia* (Bollet et al., 1995) which has emerged as an important nosocomial pathogen, especially in debilitated and immunocompromised persons (Denton and Kerr, 1998). Some species of soil origin, e.g. *Pseudomonas putida* (Magnuson et al., 1990), can be responsible for the spoilage of vegetable products during storage. The pathogenic species can be transferred to humans during vegetable consumption and some fresh-cut products have been linked to food-borne outbreaks (Sivapalasingam et al., 2004; DeWaal and Bhuiya, 2007).

In order to increase yield and quality, alternative agricultural systems like soilless cultivation systems (or hydroponics) consisting of growing plants in water containing mineral nutrients (nutrient

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solution) have been tested for many vegetables. Soil may be replaced by mineral or organic materials that support the roots while nutrients are diffused through the nutrient solution. In these conditions, the vegetables are cleaner and have higher sanitary quality than those from traditional (in soil) cultivation systems (Orozco et al., 2008; Selma et al., 2012) as there is no contact between soil and produce. Among soilless cultivation techniques, the floating system is a cheap and easy culture system where plants are held on panels that float on the nutrient solution.

The radish (*Raphanus sativus* L.) is a member of the Brassicaceae family grown and consumed throughout the world and its request is on the increase (Salerno et al., 2005). The edible part of this vegetable is the swollen root (derived from hypocotyl and upper radicle tissues) that can be harvested within four to six weeks from planting. Radishes are usually eaten raw for their crisp texture and pungent, peppery flavour and are also appreciated for its content of ascorbic acid, phenolic acids, anthocyanins and glucosinolates that can have a positive effect on consumer's health (Giusti and Wrostald, 1996; Lu et al., 2008; Jing et al., 2012).

Enterobacteria and *Pseudomonas* spp. have been identified within the total microbial population of radish sprouts grown in soil (Skowronek et al., 1998), confirming that radishes, if not properly treated before eating, may contribute to the spread of undesired bacteria. Islam et al. (2004) stated that a one-time application of contaminated (*Salmonella*) irrigation water or compost can result in the contamination of radishes and carrots well beyond their growing cycle.

The cultivation on floating panels is easy to apply to radishes and allows their production in an almost ready-to-eat form, since substrate and/or pesticide residues are absent and a single washing treatment could be enough before eating. Nevertheless, mineral nutrient solution, as irrigation water in soil, might play an important role in the pathogenic contamination of the plants. Hence, the objectives of the present study were: to investigate the survival of five species of Enterobacteriaceae family, *L. monocytogenes*, *S. maltophilia* and *P. putida* in mineral nutrient solution during the whole soilless crop cycle of the radish; to monitor their transference to the hypocotyl; to evaluate their viability on the radish surface at harvest; and to examine their internalization in the plants.

2. Materials and methods

2.1. Plant material and microbial strains

Radish (*R. sativus* L.) seeds cultivar Saxa 2 were purchased from Euroselect (Bari, Italy). Sterilized sand was used as a medium to germinate seeds and grow plantlets till full cotyledon development. The sand was moistened with deionized water and placed in a 4-cm layer in a glass tray. Radish seeds were placed on the surface, covered with a 0.5-cm layer of sand, and kept in the dark at 20 °C until cotyledon emergence.

Citrobacter freundii PSS60, *Enterobacter* spp. PSS11, *Escherichia coli* PSS2, *Klebsiella oxytoca* PSS82, *Serratia grimesii* PSS72, *P. putida* PSS21 and *S. maltophilia* PSS52 of food origin (Todaro et al., 2011), belonging to the culture collection of the Agricultural Microbiology laboratory of DEMETRA Department — University of Palermo (Palermo, Italy), were propagated in Nutrient Broth (NB) (Difco Laboratories, Detroit, MI). Except *P. putida* PSS21, incubated at 20 °C for 24 h, the other cultures were incubated at 37 °C for 24 h. *L. monocytogenes* ATCC 19114[T] was propagated in Brain Heart Infusion (BHI) (Oxoid, Basingstoke, England) at 37 °C for 24 h.

2.2. Experimental plan

Ten trials were followed in this work (Fig. 1). Mineral nutrient solution (MNS), prepared using ground water treated with inverse osmosis (electrical conductivity 430 μS/cm; pH 7.7), contained NO_3^-

(20.00 mmol/L), NH_4^+ (1.25 mmol/L), $H_2PO_4^-$ (2.00 mmol/L), K^+ (11.00 mmol/L), Ca^{2+} (5.75 mmol/L), Mg^{2+} (1.00 mmol/L), SO_4^{2-} (2.30 mmol/L), Fe^{3+} (18 μmol/L), Mn^{2+} (9 μmol/L), BO_3^{3-} (9 μmol/L), Zn^{2+} (3.8 μmol/L) and MoO_4^{2-} (3.6 μmol/L). The electrical conductivity (EC) of the MNS was 2.5 mS/cm and the pH was 6.0. Fresh microbial cultures were prepared as follows: centrifuged at $10.000 \times g$ for 5 min, washed in Ringer's solution (Sigma-Aldrich, Milan, Italy) and re-suspended in the same solution till reaching an optical density (OD) of ca. 1.00, measured by 6400 Spectrophotometer (Jenway Ltd., Felsted Dunmow, UK) at 600 nm wavelength, which approximately corresponds to a concentration of 10^9 CFU/mL. Plastic hydroponic boxes (13.5 cm long × 9.5 cm wide × 6.0 cm deep), each designed to hold 6 plants in the holes of the lid (about 468 plants/m^2), were sterilized by autoclaving at 121 °C for 20 min before filling with 650 mL of non-sterilized MNS. Cell suspensions (Cf, *C. freundii* PSS60; En, *Enterobacter* spp. PSS11; Ec, *E. coli* PSS2; Ko, *K. oxytoca* PSS82; Sg, *Ser. grimesii* PSS72; Pp, *P. putida* PSS21; Sm, *S. maltophilia* PSS52; Lm, *L. monocytogenes* ATCC 19114[T]) were added singly till a final concentration of approximately 10^6 CFU/mL; a vigorous mixing was performed by means of a sterile pipette and the boxes were closed with a plastic cover with six holes. Two control conditions were included in the system: Ctrl, non-sterilized MNS non-inoculated; and sterilized Ctrl (SCtrl), filter (0.20-μm pore size filter, Sartorius, AubagneCedex, France) sterilized MNS non-inoculated. Two boxes were prepared for each trial and two independent experiments were performed in two consecutive days.

Ten days after sowing (end of March 2012), the radish plantlets with fully developed cotyledons were picked up and before immersion in MNS, sand was removed from the roots by washing with sterile water. Plants were then plugged into the holes of the lid and tightly fastened with non-absorbent cotton, so that the roots were below the cover and the hypocotyls and the cotyledons were above the cover. All 20 boxes of each repetition were moved to an unheated plastic greenhouse (SAGA Department — University of Palermo, Italy), where the radish plants were grown during spring 2012. The MNS was monitored daily for water consumption and weekly for EC and pH. Hydroponic boxes were replenished with new MNS when the volume of MNS dropped below 500 mL.

In order to evaluate the behavior in MNS without the influence of other variables, the eight pure cultures, after washing, were also inoculated (at the final concentration reported above) in sterile test tubes containing filter sterilized MNS and kept in the greenhouse for the entire radish growth cycle.

2.3. Sample collection

The sampling plan included both MNS and plants:

a) MNS (1 mL) was sampled, in duplicate for each trial, at the time of cell suspension addition (T_0) and at 7-day intervals, after thorough agitation with a sterile pipette. Each sample was collected aseptically with a portable Bunsen and a sterile pipette and transferred into a sterile test tube;

b) Plants were collected at harvest with sterile dissecting scissors and tweezers used to transfer the hypocotyls into sterile plastic bags. Two hypocotyls (one from each box) were collected from each trial for direct microbial count and two other hypocotyls were collected for the internal detection of bacteria.

The pure cultures were transferred to a sterile hood and, soon after collection (1 mL), brought back to the greenhouse.

2.4. Microbiological analysis

Microbiological analyses were performed after decimal serial dilution of pure cultures, MNS and radish samples in Ringer's solution. Radishes (approximately 10 g) were homogenized in a stomacher (BagMixer® 400, Interscience, Saint Nom, France) for 2 min at the
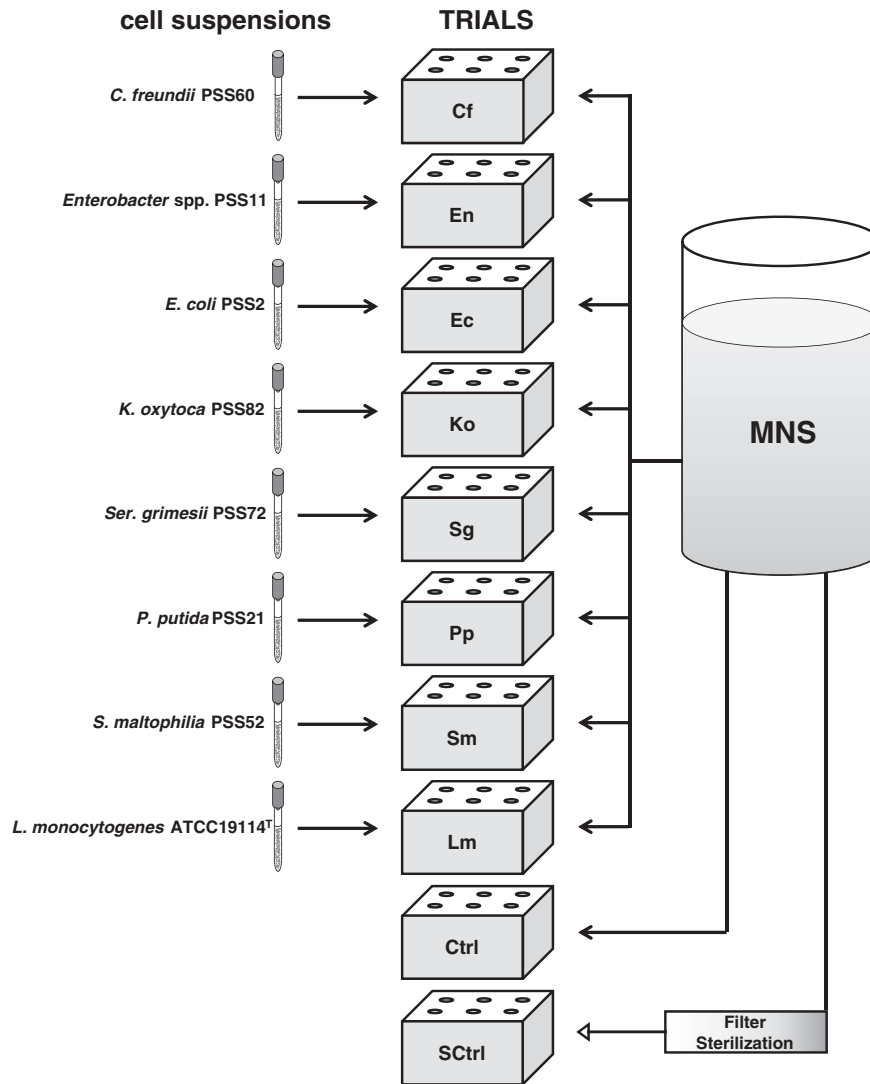
cell suspensions   **TRIALS**

Fig. 1. Graphical representation of the inoculation scheme of mineral nutrient solution (MNS) with spoilage and pathogenic bacteria.

highest speed. Microbial suspensions were plated and incubated as follows: total mesophilic count (TMC) on Plate Count Agar (PCA), incubated aerobically at 30 °C for 72 h for all trials; Enterobacteria (trials Cf, En, Ec, Ko, Sg, Ctrl and SCtrl) on Violet Red Bile Glucose Agar (VRBGA) incubated aerobically at 37 °C for 24 h; *P. putida* (trials Pp, Ctrl and SCtrl) on *Pseudomonas* Agar Base (PAB) supplemented with 10 mg/mL cetrimide fucidin, incubated aerobically at 20 °C for 48 h; *L. monocytogenes* (trials Lm, Ctrl and SCtrl) on *Listeria* Selective Agar Base (LSAB) supplemented with SR140 (Oxford formulation), incubated aerobically at 37 °C for 48 h; and *S. maltophilia* (trials Sm, Ctrl and SCtrl) on vancomycin–imipenem–amphotericin B (VIA) agar (Kerr et al., 1996), incubated aerobically at 30 °C for 48 h. Microbiological counts were performed in duplicate.

All media and supplements were purchased from Oxoid.

Analysis of variance (ANOVA), elaborated with the program SPSS 14.0 (SPSS Inc., Chicago, IL, USA), was performed. Significance level P<0.05. Tukey–Kramer's multiple range test was used to determine differences among microbial population on the plants of the different trials.

2.5. Recognition of bacterial strains

Several colonies developed onto the surface of the specific media (VRBGA, PAB, LSAB and VIA) at the highest dilution of MNS and hypocotyls from the inoculated trials were picked up from agar plates and cultured in NB (trials Cf, En, Ec, Ko, Sg, Pp and Sm) or BHI (trial Lm). Except cells from MNS and hypocotyls of the trial Pp, incubated overnight at 20 °C, all other cells were let to grow overnight at 37 °C. The cultures were purified to homogeneity by successive sub-culturing onto Nutrient Agar (NA) or solid BHI (1.6 g/L agar) in the optimal growth conditions and kept at −80 °C in glycerol (20%, v/v) stocks.

The isolates and the eight pure cultures were analyzed by randomly amplified polymorphic DNA-PCR (RAPD-PCR). DNA from broth cultures was extracted by the Instagene Matrix kit (Bio-Rad, Hercules, CA) as described by the manufacturer. Crude cell extracts were used as templates for PCR reactions. RAPD-PCR was performed by means of T1 Thermocycler (Biometra, Göttingen, Germany) using the primer M13 (Stenlid et al., 1994). Amplifications were performed applying the conditions reported by Zapparoli et al. (1998). PCR products were separated by electrophoresis on 1.5% (w/v) agarose gel (Gibco BRL, Cergy Pontoise, France) and visualized by UV transillumination after staining with SYBR® safe DNA gel stain (Molecular Probes, Eugene, OR). GeneRuler 100 bp Plus DNA ladder (M·Medical Srl, Milan, Italy) was used as a molecular size marker.

2.6. Denaturing gradient gel electrophoresis (DGGE) analysis

At harvest, MNS samples were analyzed by DGGE. One hundred milliliters of each MNS was centrifuged at 10.000×g for 5 min and

the pellet was used for total DNA extraction performed with the FastDNA® Pro Soil-Direct Kit (MP Biomedicals, CA, USA) following manufacturer's instructions. Bacterial DNA was amplified with the primer pair GC-HDA1/HDA2 (Walter et al., 2000). PCRs were carried out as reported by Giannino et al. (2009). The amplicons were run in polyacrilamide gels (20 cm×20 cm×1 mm) by means of the Phor-U2 system (Ingeny, Leiden, The Netherlands). Gels were prepared with 6% (w/v) polyacrylamide [acrylamide/bisacrylamide (37:1)] in a 1× TAE [40 mmol/L Tris, 20 mmol/L acetic acid, and 1 mol/L EDTA (pH 8.0)] buffer with a 30–60% denaturing gradient [100% denaturant corresponding to 7 mol/L urea and 40% (v/v) deionised formamide] increasing in the direction of electrophoresis. Electrophoresis was performed in 1× TAE at 60 °C for 14 h. After run, gels were stained in a $AgNO_3$ (0.2%, w/v) solution (Niessen et al., 2005) and captured by Gel Doc 2000 (Bio-Rad).

2.7. Evaluation of bacterial internalization

In order to investigate the internal presence of the added bacterium in the hypocotyl of the corresponding trial, at harvest, two radishes for each of the eight inoculated trials were superficially sterilized. The following procedure was developed by modification of the method of Hata et al. (2002): washing with sterile distilled H_2O (sd-H_2O); 5 min contact with NaClO 5% (v/v); washing with sd-H_2O; 90 s contact with ethanol 95% (v/v); washing with sd-H_2O; 3 min contact with H_2O_2 5% (v/v); washing with sd-H_2O; 3 min contact with streptomycin 0.005% (w/v)–cycloeximide 0.1% (w/v); and final washing with sd-H_2O. In order to verify the efficacy of the surface decontamination procedure applied, the treated radishes were cut into four wedges and laid (15 min) onto PCA medium so that almost the entire surface was in contact with the nutrients; petri dishes were incubated at 30 °C for 72 h and observed for colony development.

Surface sterile radishes were transferred into sterile bags to be homogenized after addition of the enrichment broth medium (final ratio 1:10) optimal and specific for the growth of each bacterium object of the search: Enterobacteria Enrichment Broth (EEB, OXOID) for trials Cf, En, Ec, Ko and Sg; *Pseudomonas* Medium (PM) (gelatine peptone 16 g/L, casein hydrolysate 10 g/L, potassium sulfate 10 g/L, magnesium chloride 1.4 g/L) for trial Pp; Half Fraser Broth (HFB, OXOID) for trial Lm; and VIA broth for trial Sm. Non-surface sterilized radishes were subjected to the same enrichment procedure as control for each trial. The incubation was for three days at the optimal growth temperature. The broths with a visible turbidity were streaked onto the corresponding agar media as reported above for plate counts.

The analyses were carried out in duplicate.

3. Results

3.1. Radish growth

The establishment of the plants was close to 100%. After one week from transplant in the hydroponic boxes, plants started the thickening growth of the hypocotyl and after four weeks they reached the commercial size (about 2.5 cm on average) and were harvested. During plant growth the average temperature of air and MNS was 21.5 °C and 23.4 °C, respectively. Air temperature ranged between 32.1 °C (day) and 11.7 °C (night), while MNS temperature had a wider range, 38.6 °C (day)–8.8 °C (night). The MNS used by each plant till complete hypocotyl development was about 315 mL. Due to water absorption and evaporation and to MNS replenishing, the characteristics of MNS in the boxes varied during plant growth. The EC and pH of MNS (on average 2.93 mS/cm and 6.08, respectively) increased slightly after two weeks of cultivation, but reached 3.52 mS/cm and 6.82 at harvest. The highest values were recorded at the fourth week, when the MNS before replenishing had an EC of 4.47 mS/cm and the pH

was 7.40. No significant differences were found among MNS of the different trials.

3.2. Microbiological analysis

3.2.1. Behavior of inoculums in sterile MNS

The evolution of pure cultures of *C. freundii* PSS60, *Enterobacter* spp. PSS11, *E. coli* PSS2, *K. oxytoca* PSS82, *Ser. grimesii* PSS72, *P. putida* PSS21, *S. maltophilia* PSS52 and *L. monocytogenes* ATCC 19114[T] was evaluated in sterile MNS in test tubes incubated in the same environmental conditions of the radish trials (Fig. 2A–H). The initial level was in the range 6.90–7.58 Log CFU/mL. Although a low increase in concentration was registered for all eight bacteria at 24 h from inoculation, a general decrease in level was observed during incubation. After seven days, the concentrations were about three Log cycles lower than T_0, till reaching less than 0.50 Log CFU/mL for *C. freundii* PSS60 and *K. oxytoca* PSS82, almost 1–1.5 Log CFU/mL for the other enterobacteria and little more than 2 Log CFU/mL for the rest of the test bacteria at the 28th day of observation.

3.2.2. Analyses of MNS

The microbial counts of MNS during the entire growth cycle of the radish from different trials are graphically reported in Fig. 2(A–L). At the beginning of the observation, the inoculated trials contained a TMC ranging between 6.69 and 7.78 Log CFU/mL, while Ctrl and SCtrl showed an initial TMC of 4.39 and 0.97 Log CFU/mL, respectively. In general, all inoculated trials showed similar levels of TMC during the 28 days of the plant growth cycle and, at the end of the period of observation, they were almost 1 Log cycle lower than those estimated at T_0. Interestingly, TMC of both control trials was similar and superimposable on those displayed by the inoculated trials (P > 0.05) from the seventh day till the end of the experimentation.

The counts conducted with the media specific for the bacteria inoculated showed a general trend of concentration reduction. The minimum level was registered for trial Pp after 14 days from inoculation, for trials Ec and Ko at the 28th day, while for the other trials at the 21st day of experimentation. At harvest, the five trials with enterobacteria displayed concentrations of maximum 3.01 Log CFU/mL onto VRBGA, while higher counts were observed for trials Pp, Sm and Lm onto PAB, VIA and LSAB, respectively. Both control trials analyzed on the same four specific media showed similar behavior onto PAB and VIA, from the beginning till the end of the radish growth, while a different observation was made onto LSAB and VRBGA. Presumptive listerias were estimated in trial Ctrl since T_0, whereas in trial SCtrl from the 14th day onward; presumptive enterobacteria ranged between 0.94 (T_0) and 1.74 (28th day) Log CFU/mL in trial Ctrl, but were never detected in SCtrl. The direct comparison of data registered on the specific media from the inoculated trials with those from Ctrl and SCtrl, except in case of trial Pp, showed that the concentration levels estimated onto VRBGA, VIA and LSAB were only due to the inoculated bacteria, excluding the interference by the microorganisms present in MNS added daily. Regarding trial Pp, MNS seemed to have influenced the counts observed on PAB.

3.2.3. Analyses of plants

At harvest, plants were microbiologically investigated for TMC and for the presence of the bacteria inoculated in the different trials (Table 1). TMC values did not show significant differences among the different trials, including controls; they ranged between 6.44 and 7.24 CFU/g. Also the counts estimated onto the specific media for the inoculated trials were not statistically different from those registered for the control trials and the highest levels of concentration (5.95–6.25 CFU/g) were registered on PAB.
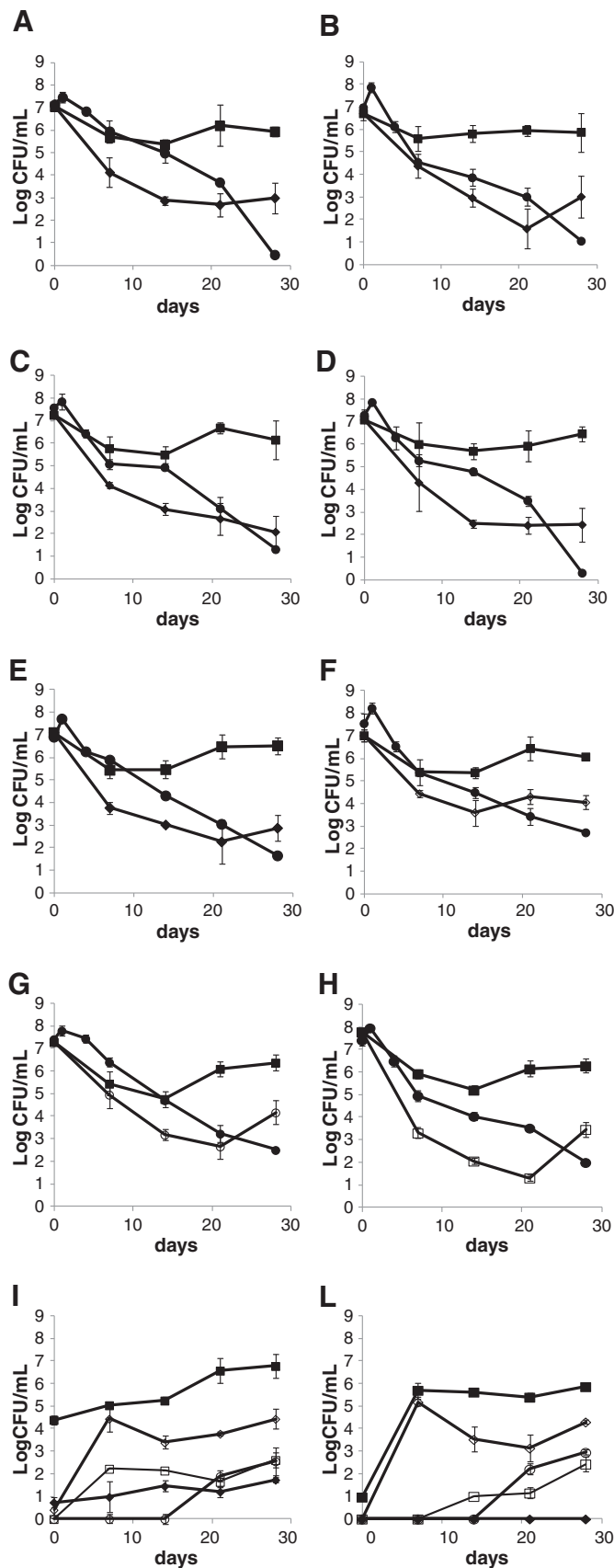
Table 1
Microbial load (Log CFU/g) of radish samples at harvest.

Trials	Media				
	PCA	VRBGA	PAB	VIA	LSAB
Cf	6.86 ± 0.28a	4.55 ± 0.22a	n.d.	n.d.	n.d.
En	6.76 ± 0.53a	5.05 ± 0.27a	n.d.	n.d.	n.d.
Ec	7.24 ± 0.28a	4.81 ± 0.26a	n.d.	n.d.	n.d.
Ko	6.70 ± 0.16a	3.92 ± 0.87a	n.d.	n.d.	n.d.
Sg	6.44 ± 0.11a	4.78 ± 0.15a	n.d.	n.d.	n.d.
Pp	6.98 ± 0.46a	n.d.	6.25 ± 0.22a	n.d.	n.d.
Sm	6.78 ± 0.22a	n.d.	n.d.	4.47 ± 0.27a	n.d.
Lm	7.13 ± 0.98a	n.d.	n.d.	n.d.	3.57 ± 0.42a
Ctrl	6.61 ± 0.48a	4.08 ± 0.75a	5.95 ± 0.82a	3.85 ± 0.66a	2.57 ± 0.81a
SCtrl	7.23 ± 0.20a	4.06 ± 0.91a	6.39 ± 0.07a	3.82 ± 0.83a	3.13 ± 0.18a

Abbreviations are as follows: Cf, mineral nutrient solution (MNS) inoculated with *C. freundii* PSS60; En, MNS inoculated with *Enterobacter* spp. PSS11; Ec, MNS inoculated with *E. coli* PSS2; Ko, MNS inoculated with *K. oxytoca* PSS82; Sg, MNS inoculated with *Ser. grimesii* PSS72; Pp, MNS inoculated with *P. putida* PSS21; Sm, MNS inoculated with *S. maltophilia* PSS52; Lm, MNS inoculated with *L. monocytogenes* ATCC 19114[T]; Ctrl, MNS non-inoculated (control trial); SCtrl sterilized MNS non-inoculated (sterilized control trial). PCA, plate count agar; VRBGA, violet red bile glucose agar; PAB, *Pseudomonas* agar base; VIA, vancomycin-imipenem-amphotericin B agar; and LSAB, *Listeria* selective agar base.
Results indicate mean value ± SD of four replicates of two independent experiments. Values within the same column followed by the same letter are not statistically different at P<0.05 (Tukey–Kramer's multiple range test).
n.d. not determined.

3.3. Bacterial comparison

The isolates collected from VRBGA, PAB, LSAB and VIA resulting from plate count of MNS of the inoculated trials were analyzed at strain level by means of RAPD-PCR with primer M13. Amplified DNAs from the isolates of a given trial, together with that of the pure culture corresponding to the same trial, were loaded on a gel in order to recognize the added bacteria through the radish growth cycle and to evaluate their contribution to the plate counts. The direct comparison of the RAPD patterns allowed the recognition and monitoring of the added cultures during the entire experimentation (Fig. 3A). In all eight trials, although at decreasing levels, the inoculums were confirmed to persist in MNS in a living state till the 28th day of observation. The concentration levels estimated onto the specific media were found to be exclusively due to the inoculated bacteria. Furthermore, in case of trial Pp (Fig. 2F), although the pseudomonads vehiculated by MNS (Fig. 2I) contributed to the final concentration counted onto PAB, RAPD profile comparison clearly recognized *P. putida* PSS21 at the highest dilution of cell suspension.

The isolates from the plate counts of the hypocotyls, at the highest dilutions, were collected and analyzed by RAPD-PCR as reported for MNS isolates. None of the isolates of any inoculated trial were found to share the same RAPD profile of the added bacteria (Fig. 3B). These results cannot exclude the presence of the bacteria in the mature hypocotyls, but clearly showed that the test organisms were not dominant in the edible part of the radishes.

Fig. 2. Microbial counts of mineral nutrient solution (MNS). A, MNS inoculated with *C. freundii* PSS60 (trial Cf); B, MNS inoculated with *Enterobacter* spp. PSS11 (trial En); C, MNS inoculated with *E. coli* PSS2 (trial Ec); D, MNS inoculated with *K. oxytoca* PSS82 (trial Ko); E, MNS inoculated with *Ser. grimesii* PSS72 (trial Sg); F, MNS inoculated with *P. putida* PSS21 (trial Pp); G, MNS inoculated with *S. maltophilia* PSS52 (trial Sm); H, MNS inoculated with *L. monocytogenes* ATCC 19114T (trial Lm); I, MNS non-inoculated (trial Ctrl); L, sterilized MNS non-inoculated (trial SCtrl). Symbols: ●, pure culture; ■, PCA, plate count agar; ◆, VRBGA, violet red bile glucose agar; ◇, PAB, *Pseudomonas* agar base; ○, VIA, vancomycin-imipenem-amphotericin B agar; □, LSAB, *Listeria* selective agar base. Data represent the mean of four replicates of two independent experiments. Bars represent standard deviation of the mean. Vertical bars not visible are smaller than symbol size.
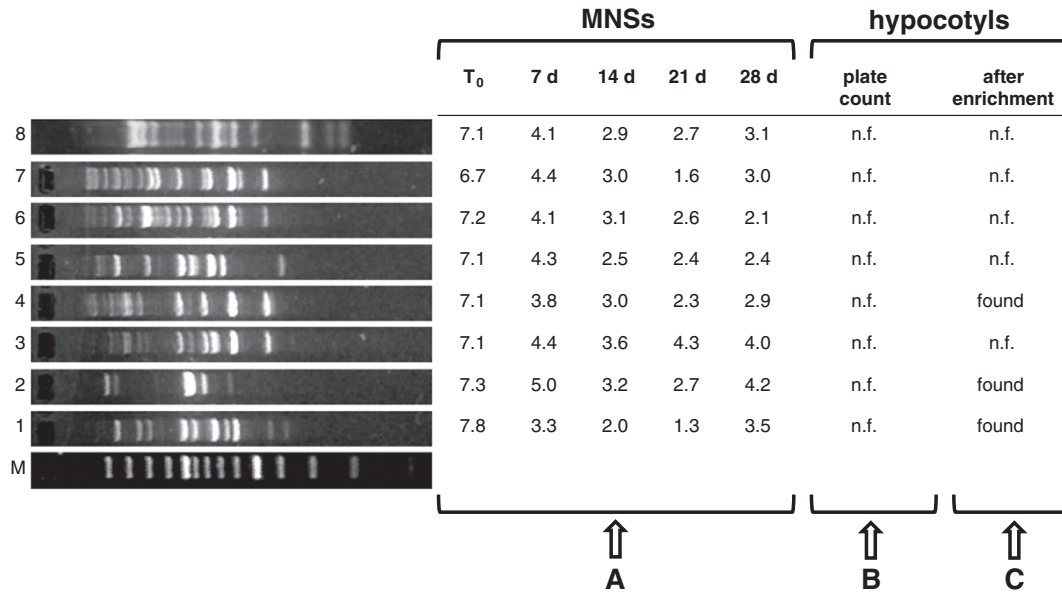
	MNSs					hypocotyls	
	T_0	7 d	14 d	21 d	28 d	plate count	after enrichment
8	7.1	4.1	2.9	2.7	3.1	n.f.	n.f.
7	6.7	4.4	3.0	1.6	3.0	n.f.	n.f.
6	7.2	4.1	3.1	2.6	2.1	n.f.	n.f.
5	7.1	4.3	2.5	2.4	2.4	n.f.	n.f.
4	7.1	3.8	3.0	2.3	2.9	n.f.	found
3	7.1	4.4	3.6	4.3	4.0	n.f.	n.f.
2	7.3	5.0	3.2	2.7	4.2	n.f.	found
1	7.8	3.3	2.0	1.3	3.5	n.f.	found
M							
	A					B	C

Fig. 3. Bacterial recognition by means of RAPD-PCR analysis in mineral nutrient solution (MNS) during radish growth (A) and in hypocotyls, at harvest, before (B) and after (C) enrichment. Lanes: M, marker; 1, *C. freundii* PSS60; 2, *Enterobacter* spp. PSS11; 3, *E. coli* PSS2; 4, *K. oxytoca* PSS82; 5, *Ser. grimesii* PSS72; 6, *P. putida* PSS21; 7, *S. maltophilia* PSS52; 8, *L. monocytogenes* ATCC 19114[T]. The maximum level of detection (Log CFU/mL) of the single inoculums in MNS is reported for each collection time. Abbreviations: n.f., not found.

3.4. Culture-independent analysis of MNS

DGGE analysis was applied on total DNA extracted from MNS at the time of radish harvest to better investigate the microbial communities developed in the experimental hydroponic systems. DNAs from pure cultures were also loaded onto the gel for direct comparison (Fig. 4). Only *Enterobacter* spp. PSS11 and *L. monocytogenes* 19114[T] were characterized by a single band, while the other six cultures produced multiple bands. DGGE clearly revealed the presence of *P. putida* PSS21 in trial Pp, at the end of experimentation, while the other seven bacteria were not detected. Moreover, the bands ascribable to *Pseudomonas* spp. were found in all inoculated trials, as well as in the control trials. Hence, the combination of data from DGGE and plate counts confirmed that MNS influenced the levels of count of pseudomonads.

3.5. Analysis of internalized bacteria

The internal presence of the added bacterium in the hypocotyls of radishes was evaluated at harvest. The radishes were surface sterilized with a protocol developed in this study. The efficacy of the surface decontamination protocol was tested by direct contact of the radish surfaces of all trials with an agar medium. The validation occurred since no colony developed after incubation.

Surface sterilized radishes were subjected to enrichment after homogenization. In these conditions, only the internal microorganisms able to grow in the specific liquid media determined the turbidity of the broths. All eight enrichment broths showed a clear microbial growth after three days of incubation and, for this reason, were subsequently streaked onto the specific agar media. The colonies developed were subjected to RAPD analysis and the comparison with the pure cultures was performed as reported above. *C. freundii* PSS60, *Enterobacter* spp. PSS11 and *K. oxytoca* PSS82 were clearly recognized, while the other five bacteria were not found (Fig. 3C). The same results were obtained with the non-surface sterilized radishes, confirming that *E. coli* PSS2, *Ser. grimesii* PSS72, *P. putida* PSS21, *S. maltophilia* PSS52 and *L. monocytogenes* ATCC 19114[T] were not present, at least in a living form, in the mature hypocotyls.
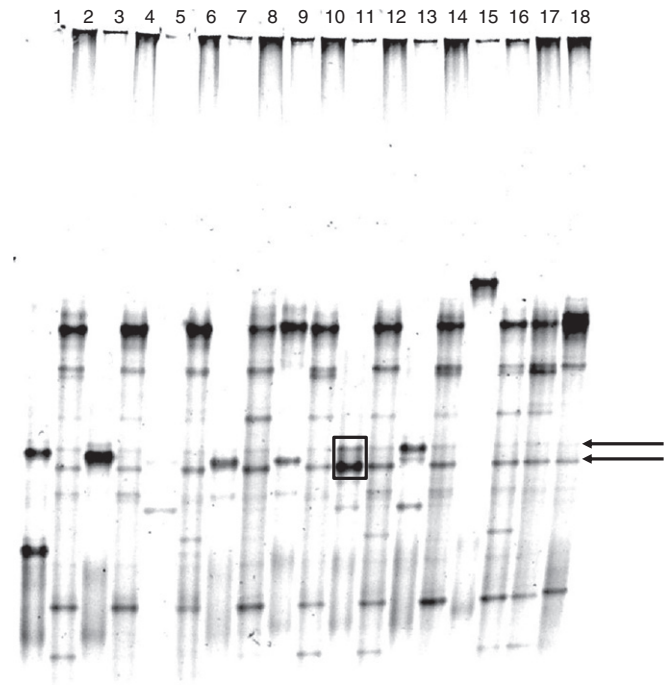

Fig. 4. Denaturing gradient gel electrophoresis (DGGE) profiles of pure cultures and mineral nutrient solution (MNS) samples at radish harvest. Lanes: 1, *C. freundii* PSS60; 2, trial Cf, MNS inoculated with *C. freundii* PSS60; 3, *Enterobacter* spp. PSS11; 4, trial En, MNS inoculated with *Enterobacter* spp. PSS11; 5, *E. coli* PSS2; 6, trial Ec, MNS inoculated with *E. coli* PSS2; 7, *K. oxytoca* PSS82; 8, trial Ko, MNS inoculated with *K. oxytoca* PSS82; 9, *Ser. grimesii* PSS72; 10, trial Sg, MNS inoculated with *Ser. grimesii* PSS72; 11, *P. putida* PSS21; 12, trial Pp, MNS inoculated with *P. putida* PSS21; 13, *S. maltophilia* PSS52; 14, trial Sm, MNS inoculated with *S. maltophilia* PSS52; 15, *L. monocytogenes* ATCC 19114[T]; 16 trial Lm, MNS inoculated with *L. monocytogenes* ATCC 19114[T]; 17 trial Ctrl, MNS non-inoculated (control trial); 18, trial SCtrl, sterilized MNS non-inoculated (sterilized control trial). The arrows and the box indicate the two major bands of the species *P. putida*.

4. Discussion

The biological safety of foods, defined as "biosafety", refers to the analyses of the biological hazards, including bacteria, viruses, parasites, biotoxins and prions, which represent serious risks to public health. The term was coined in the 1970s after reports about the high incidence of laboratory-acquired infections among clinical and medical research laboratory staff (Pike, 1976). Good biosafety practices on farms, eradication and surveillance programs are being implemented in EU member Countries (European Commission, 2011). The effect of bacterial pathogen contamination on vegetables eaten raw has been recently recorded with *E. coli* O124 in Northern Europe (Moulson, 2011). That event highlighted the importance of the safety of raw vegetables, especially when they are cultivated under organic regimes.

In this work, we investigated the hygienic safety of radishes grown hydroponically in MNS artificially contaminated with food spoilage and pathogenic bacteria. The levels of inoculation were around 10^7 CFU/mL to simulate a massive contamination of MNS during the first stages of plant development. *R. sativus* was chosen not only for its short crop cycle, during which the eight spoilage/pathogen bacteria (*C. freundii*, *Enterobacter* spp., *E. coli*, *K. oxytoca*, *Ser. grimesii*, *P. putida*, *S. maltophilia* and *L. monocytogenes*), representative of several microbial groups affecting the safety and the microbiological stability of vegetables, may presumably persist at consistent concentrations, but also because it does not undergo a thermal treatment and a contamination of the produce may be dangerous for consumers. Furthermore, these plants have been tested widely in soilless cultivation on floating panels to obtain products with enhanced quality (Nicola et al., 2005; Salerno et al., 2005; Zanin et al., 2009) that need minimal sanitizing treatments before consumption.

The work was carried out with a MNS suitable for soilless cultivation of radish on floating panels. Once the plantlets were put in the boxes containing the different inoculums in MNS, they were placed in a greenhouse during the spring season, at an average air and MNS temperature of about 21.5 and 23.4 °C, respectively. The plants showed fast and regular growth, and no influence of the inoculated bacteria was recorded on hypocotyls development.

In order to evaluate the influence of the microbial load of the MNS added periodically to bring the growth medium to the initial volume and the contamination by the environment, two controls (un-inoculated) were followed, one in non-sterile MNS and another one in sterile MNS. Our data showed that presumptive enterobacteria, *Listeria* spp. and *Stenotrophomonas* spp. present in MNS did not affect the counts of these microbial groups, since in both controls their concentration levels, obtained on the specific media, were lower than those registered for the inoculated trials during the entire cycle of radish growth. However, the presence of pseudomonads in Ctrl and SCtrl was in the same order of magnitude of that estimated for trial Pp, showing a possible influence of MNS on the counts registered on PAB from the seventh day onward.

In general, all inoculated trials showed similar levels of TMC in MNS during the 28 days of the plant growth cycle, even though the counts conducted with the media specific for the inoculated bacteria decreased. At harvest, the minimum level was registered in MNS for the five trials with enterobacteria. Very few works have been conducted on the investigation of the survival of undesired food bacteria in hydroponic systems. Guo et al. (2002) found that, starting from 4.55 Log CFU/mL, the size of salmonellae in inoculated half-strength Hoagland solution used for the cultivation of tomato plants was 4.46 to 4.65 Log CFU/mL throughout the 9-day period of observation. In our study, starting from inoculation levels of about 10^7 CFU/mL in MNS where radish roots were growing, data taken after seven days showed, for all eight bacteria object of study, levels of concentration similar to those reported by Guo et al. (2002), whereas higher levels were found for the pure cultures of the same

eight organisms in sterile MNS in test tubes, a condition that excluded the interactions with the other microorganisms hosted in non-sterile MNS.

The persistence of the strains added was monitored during the experimentation by RAPD-PCR. The isolates taken from a specific medium at the highest dilution were analyzed and compared with the pure strain of the corresponding trial. The direct comparison of the RAPD patterns allowed the recognition and monitoring of the added cultures during the entire growth cycle of radish. This strategy also confirmed that, except in the case of trial Pp, the concentration levels estimated onto the specific media were exclusively due to the inoculated bacteria. The pseudomonads present in MNS contributed to the final concentration of trial Pp, but *P. putida* PSS21 inoculated at T_0 was clearly recognized at the 28th day of experimentation. The high counts estimated on PAB in both control trials since the seventh day are not surprising in this cultivation system. Fluorescent *Pseudomonas* species and *P. putida* were the major isolates belonging to γ-Proteobacteria in hydroponic cultures of moss *Racomitrium japonicum* (Tani et al., 2011). Furthermore, many *Pseudomonas* species were also found in the bacterial communities of the filter units employed for controlling plant pathogens in the recycled solutions used in soilless cultures (Renault et al., 2012), confirming that pseudomonads are ubiquitous in these environments. Also the finding that PAB counts in SCtrl, from 0 at T_0, reached more than 10^5 CFU/mL after seven days is not anomalous, since pseudomonads are commonly found associated to plants (Naik et al., 2008). Thus, they might have been transported to the sterile trial by plantlet roots.

DGGE analysis was useful to better evaluate data obtained with the culture-dependent methodology. Only two pure strains generated single bands, while multiple bands were displayed by the rest of the cultures employed. In general, multiple-band formation may complicate the interpretation of DGGE patterns, but in our study, this phenomenon did not interfere with the recognition of the bacterial species in MNSs. A multiple-band DGGE fingerprint for a pure strain could indicate multiple 16S rRNA gene copies with sequence divergence (Leys et al., 2004). Further molecular analyses are necessary to explain the multiple-band DGGE pattern formation of *C. freundii* PSS60, *E. coli* PSS2, *K. oxytoca* PSS82, *Ser. grimesii* PSS72, *P. putida* PSS21 and *S. maltophilia* PSS52.

DGGE applied on MNS at harvest showed that only the presence of *P. putida* PSS21 was clearly ascertained in trial Pp. The culture-independent detection was not effective for the individuation of the other seven bacteria in the corresponding trials, although, as confirmed by RAPD analysis, they were isolated from the specific media. Other authors (Shinohara et al., 2011) reported that some bacteria isolated by means of dilution plating on specific media were not detected through DGGE analysis based on 16S rRNA gene, suggesting that those isolates did not constitute the major components of the microbial community investigated. In our study, TMC estimated at day 28 for MNS of the different trials was approximately 10^6 CFU/mL, while the counts detected using the specific media were no more than 10^3 CFU/mL for enterobacteria, little more than 10^3 CFU/mL for *L. monocytogenes* ATCC 19114^T and reached 10^4 CFU/mL with *P. putida* PSS21 and *S. maltophilia* PSS52. Since the DGGE detection limit is in the range of 1–10% of the total population (Muyzer and Smalla, 1998), both *P. putida* PSS21 and *S. maltophilia* PSS52 should have been found easily, but the latter was not detected. This result may be explained with the fact that, depending on the liquid substrate and primers employed different DGGE detection limits may be found for different bacteria (Bester et al., 2010). DGGE patterns also revealed the presence of bands ascribable to *Pseudomonas* spp. in all inoculated and un-inoculated trials. This finding demonstrated unequivocally that pseudomonads vehiculated by MNS influenced the levels of count detected on PAB.

Radishes were microbiologically investigated at harvest. TMC, as well as the counts estimated onto the specific media, were not statistically

different for all trials. Thus, inoculated trials produced radishes characterized by the same levels of microbial contamination of those obtained from control trials. The levels of TMC were in the range 10^6–10^7 CFU/g. Our levels of TMC are different from those available in literature for radishes. Izumi (1999) reported 4.3 Log CFU/g of total microorganisms for Japanese radish shreds, raw materials that underwent a technological treatment, while Tirranen (2008) found about 8.99 Log CFU/g of aerobic bacteria and 5.81 Log CFU/g of coliforms in the near-root zone of radishes analyzed at the technical ripeness. Compared with our data, the diverse concentrations are due to the fact that those radishes were grown and collected in conditions different from those applied in the present work.

The transference of the strains added into MNS (at T_0) was evaluated in hypocotyls (at harvest) by RAPD-PCR applied on the isolates collected from specific media at the highest dilutions. Due to the negative response of this strategy, the radishes were subsequently subjected to the enrichment procedure with and without the application of a surface sterilization treatment. In both conditions, *E. coli* PSS2, *Ser. grimesii* PSS72, *P. putida* PSS21, *S. maltophilia* PSS52 and *L. monocytogenes* ATCC 19114^T were not found, while *C. freundii* PSS60, *Enterobacter* spp. PSS11 and *K. oxytoca* PSS82 were detected, showing that these bacteria were present in the internal part of hypocotyls. With this technique, the internal presence of the other five bacteria in the radishes cannot be excluded, although the results suggested that they are not present in a living form at harvest. On the other hand, the three bacteria found in radishes in living form were present at undetectable levels by plate count. Bacterial internalization in plants is a common phenomenon and it is normally a passive process. Several enteropathogens present in the irrigation water can be taken up by the root systems and enter the edible portion of different crops (Burnett et al., 2000; Guo et al., 2002; Solomon et al., 2002; Wachtel et al., 2002).

Regarding the main objectives of this work, the conclusions drafted are as follows: by means of combination of culture-dependent and culture-independent detections, the five species of Enterobacteriaceae family, *L. monocytogenes*, *S. maltophilia* and *P. putida* were found to survive in MNS during the whole soilless crop cycle of the radish, although at several orders of magnitude lower than inoculation; at harvest, only *C. freundii* PSS60, *Enterobacter* spp. PSS11 and *K. oxytoca* PSS82 were detected in the radish hypocotyls in a living form, but at subdominant levels. Since the transference of human pathogens from the cultivation system to the final product represents an issue of importance for the consumers' health, further studies on the process of internalization of the eight undesired food bacteria object of this study in radishes are being prepared, in order to picture the moment and to reveal the mechanism of their penetration, to distinguish between live and dead cells and to estimate their effective concentrations at harvest and during conservation. This study provided evidences that hydroponics are delicate cultivation systems whose contamination by undesired microorganisms, especially pathogenic species, may affect the quality of vegetables and the safety of consumers.

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