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ORIGINAL ARTICLE

Impact of fertilization by natural manure on the microbial quality of soil: Molecular approach

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Abstract The quality of soil is strongly bound by several interactions between chemical and biological components, including microbial composition, which are a key importance for soil performance. Cultural activities have a huge induction on soil health, through both modification of physicochemical proprieties and changing on soil microbial communities. This usually affects the safety of soil, and then the crop production and water.

In the present work, the information on bacterial community composition was determined from a set of 6 soils collected from 2 farms in agricultural land of Marrakech (Morocco), one of which used poultry manure (PM) and the other cow manure (CM) as fertilizers. To profile this structure of the bacterial community Denaturing Gradient Gel Electrophoresis (DGGE) of 16S rDNA fragments has been used.

These amendments resulted in the appearance of several novel bands and different relative intensities of bands between the control station and other sites studied. The stations most affected are those receiving a supply of manure rather high, which results in an organic and bacterial load in the soil. The results showed a bacterial diversity very important indicating a fecal contamination like Bacteroides, Pseudomonas, Staphylococcus,... etc. Bacteria pertain to the phylum Firmicutes and Bacteroidetes were noted to be the dominant ribotype in amended soil.

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Moreover, this work demonstrates also the existence of pathogens strains in soil amended by poultry manure (PM) belonging to the Clostridiales order and Pseudomonadales. The pathogenic bacteria detected posing a hazard of human contagion when they are used for soil practice.

1. Introduction

Soil bacteria are necessary to agrosystems. They played key roles in soil litter formation, and degradation of numerous molecules and other processing (Wu et al., 2011). Fertilization is the commonest managing agricultural soils, and for a long time, intensive farming appealed to fertilizer to increase yields.

Among the oldest way to improve soil quality for an agricultural sustainability is by increasing organic amendment offers through the addition of manure. The most used manure was as original substrates from swine, poultry, cow or horse manure (Sen and Chandra, 2009). The addition of manures to soil is considered a suitable management application in agricultural in view of the fact that push microbial respiration, enzymatic activities and azotes mineralization rates with potential mineralization of vegetable nutriments (Eriksen, 2005), and thus raise soil richness and quality (Doran et al., 1988). Several study developed in bibliography on the benefit of using manure as a fertilizer of soil (Oehl et al., 2004).

Most origins of pathogens on cultivate land are those related to the use of animal manure, it can contain dangerous bacteria that are special to the animal group but may also contain zoonotic pathogen organisms. In addition, the overuse of animal manure can release pathogens, and other dangerous chemical compounds (De Sutter and Ham, 2005). According to Cole et al. (1999) dangerous microorganisms could be transferred from animals to humans when manure is utilized as a fertilizer for the crude consumed products and by the stream of water passing the amended soil toward the surface and ground waters.

The quantity and nature of microorganism's species as well as the number of individuals in the soil are clearly affected by various environmental stresses and by agricultural practices (Bardgett et al., 1999). The different cultivation practices create indeed environmental conditions for soil organisms favoring certain functional groups (Oehl et al., 2004). The intake of fertilizing substances therefore has a great influence on soil microbial communities.

The majority of recent studies on microbial biodiversity involving molecular biology techniques allow the identification of organisms directly from environmental samples without regard to their morphology or their stage of development. Molecular approaches have been conducted to get a real understanding of microbial community structure in complex environments like soils (Smalla et al., 2007).

There are a lot of molecular techniques for the characterization of microbial communities each with their advantages and disadvantages (Kirk et al., 2004). Recently, the polymerase chain reaction (PCR) method has permitted fast and effective characterization of bacterial diversity due to its specificity and sensibilities. PCR–DGGE is one of the most effective techniques to observe the diversity, richness and evenness of microbial community (Yu and Morrison, 2004). It also has been performed for rapid changes tracking and diagnosing of bacterial diversity of soil (Martínez-Alonso et al., 2010). This is a molecular approach for separating fragments of the same size but of different compositions in nucleotides.

In this sense, the aim of this work was to assess the effects of animals manure as soil amendments, on soil microbial biomass, enzyme activity and bacterial community structure in a semi arid agro-ecosystem. This was made by studying the 16S-rDNA diversity in soil of six farms utilize cow manure (CM) and poultry manure (PM) as fertilizer, using PCR and Denaturing Gradient Gel Electrophoresis (DGGE) technique.

2. Materials & methods

2.1. Study area

The study was conducted in the semi-aride region of Marrakech, Geumassa (31°25′58.5″N and 08°26′26.1″W) of Chichaoua Province, Morocco.

The climate of the region is typified by a warmth and arid period, mostly starting from April until September, with some onerous rain time. The average annual precipitation is about 235 mm and 17.0–28.1 °C for temperature.

Soil is generally clay brown loam (42% sand, 31% silt and 27% clay).

2.2. Samples collection and analysis

Seven sampling sites were chosen for this study. Three farms using manure from cows (CM1, CM2 and CM3) and three others using those of poultry (PM1, PM2 and PM3) and a last site as a reference (R: land in same area without agricultural activity). The 0–20 cm layer of the soil was sampled in different sites and the samples were transferred to the lab in an ice box at 4 °C.

2.2.1. Soil chemical analysis

For this analysis, a part of each sampled soils was dried at 60 °C for 24 h and the big material (>2000 mm) was eliminated by sifting. The pH was determined with a portable measuring (WTW Multiline), with 10/25 ratio of soil/water. Soil organic carbon (SOC) and total nitrogen (TN) were measured respectively by dichromate oxidation and Kjeldahl method, according to the protocols mentioned in the Official Methods of Soil Analysis developed by Aubert (1978).

2.2.2. Enzyme activities

Enzymatic analysis was conduct by β-glucosidase, phosphatase and urease activity.

Activity of β-glucosidase was determined according to a method adapted by Hayano (1973); however the determination of urease activity was conducted to the method of Kandeler and Gerber (1988).

Table 1 Oligonucleotide primers used for PCR.

Primer	Primer sequence (5′ → 3′)
341F-GC*	**5′**-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CCC TAC GGG AGG CAG CAG-**3′**
907R	**5′**-CCG TCAATT CCT TTG AGT TT-**3′**

F, Forward; R, reverse.
*GC clamp, CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG.f

Table 2 Chemical properties of manure fertilization.

	Farmers						
	R	CM1	CM2	CM3	PM1	PM2	PM3
pH	7.12 ± 0.11	5.72 ± 0.07	5.61 ± 0.14	5.44 ± 0.08	6.51 ± 0.16	6.32 ± 0.12	6.82 ± 0.09
TN (g/kg)	1.49 ± 0.03	2.01 ± 0.06	1.91 ± 0.04	2.12 ± 0.13	1.84 ± 0.15	1.69 ± 0.08	1.75 ± 0.11
TP(g/kg)	0.29 ± 0.01	0.61 ± 0.02	0.56 ± 0.03	0.64 ± 0.08	0.9 ± 0.08	0.81 ± 0.07	0.87 ± 0.09
TK(g/kg)	12.41 ± 0.09	14.08 ± 0.15	13.82 ± 0.19	14.23 ± 0.42	13.05 ± 0.12	13.17 ± 0.15	12.89 ± 0.13
TOC(g/kg)	10.15 ± 0.26	15.24 ± 0.22	14.75 ± 0.13	15.31 ± 0.25	12.63 ± 0.22	13.05 ± 0.17	12.92 ± 0.15

TN: total azotes, TOC: total organic carbon, TP: total Phosphorus, TK: total potassium.

Phosphatase activity was determined using the easy, precise and fast enzyme assay according Tabatabai and Bremner (1969).

2.3. DNA extraction and PCR amplification

DNA was extracted using UltracleanTM Soil DNA isolation kit, according the directions of the maker. For PCR, the DNA concentration was adjusted to a concentration of 10 ng/μl after measurement by Nanodrop spectrophotometer.

The variable region V3–V5 of the 16S rDNA was amplified using the universal primers 341F-GC and 907R as shown in Table 1. This set of primers was structured to be specific for many bacteria (Muyzer et al., 1993). PCRs were performed in a Thermal Cycler (TECHNE TC-5000) with 50 μL reaction mixtures (3 mM $MgCl_2$, 200 μM of each nucleotide, 1 × PCR buffer with $(NH4)_2SO_4$, 5% dimethylsulfoxide, 15 pmol of each primer, 1 U of Taq DNA polymerase, and 50–200 ng template DNA). Negative control reactions without template DNA were achieved in parallel. The quality of amplicons obtained by PCR was confirmed by electrophoresis in 1.5% agarose gels and visualization on a UV trans-illuminator.

2.4. Denaturing Gradient Gel Electrophoreis (DGGE) analyzing

PCR products were analyzed through DGGE, using a 45–65% denaturing gradient (which 100% is 7 M urea and 40% deionized formamide) in 1 mm vertical polyacrylamide gels (8% of acrylamide in 0.5 × TAE buffer). Electrophoresis was performed in a DCode™ system (Bio-Rad) using 0.5 × TAE buffer during 16 h at 75 V, with an initial step at 120 V for 15 min. The gel was then stained for 5 min in an ethidium bromide solution (5%) and then digitalization in a Molecular Imager FX™ system (Bio-Rad).

2.5. Sequencing and nucleotide sequence accession numbers

The nucleotide sequence in the bands of DGGE was excised and 20 μl sterile water was added, and kept at −80 °C for 30 min. The DNA extract was amplified again by PCR with the same primer and conditions using in this study.

The sequences determined were deposited in the GenBank database taken into account species that have 97% of resemblance at minimal to define our isolate sequences.

2.6. Statistical analysis of DGGE fingerprints

In order to compare the microbial diversity among the studied fertilization types; the Shannon index (H9) and the dominance or Simpson index (D or S) were calculated based on presence or absence of bands and their relative intensities among the samples. A popular model statistic index is the Shannon–Weiner index: $H' = -\sum pi \ln pi$, where pi is the frequency of the *i*th species.

A dendrogram was established with the similarities obtained by the Pearson correlation coefficient (95% probability).

3. Results and discussion

3.1. Soil chemical properties

Fertilization by manure has shown an important variance in soil chemical characteristics (Table 2). This fertilization was augmented significantly ($p > 0.01$) the reserves of total nitrogen, total phosphorus and TOC comparing with the reference soil (R). pH in amended soil were significantly higher than that of soil R, which probably resulting to the enrichment by cations (Murugan and Kumar, 2013). Compared with poultry manure fertilized soils, cow manure showed a significantly lower in pH value.

Application of organic manure amplified the soil organic carbon reserve. Organic manure also serves to ameliorate the physical proprieties of the soil and furnish the necessary plant nutrients. It improves cation exchange capacity and acts as a moderator factor against undesirable soil pH variations (Ojeniyi et al., 2007). According to Joergensen et al. (2010), this is due to the nutrient input; also it might be resulted by the big quantity of organic and microbial carbon added by

natural manure. Due to some reason this impute of manure explains that the TN, TP and TK in fertilized soil by organic manure were significantly higher than those of control site, which may be due to the release of these elements from manure.

3.2. Enzyme activities

According to the results shown in Table 3, adding the organic manure to the soil amplified the enzyme activities of phosphatase and β-glucosidase, in which the activities were significantly higher in the fertilized soil than in the references soil (R). However, it demonstrates that, in this work urease activity is more remarkable in the unamended soil in comparison with the amended one.

An examination of the results by ANOVA marked that only the activities of β-glucosidase were significantly allied to the kind of amendment; as well as, the evaluated enzymatic activities were statistically different.

The variances resulted to adding the organic manure to the soil were 402 and 88 μg pNP g-1h-1 for phosphatase; 75 and 41 μg pNP g-1h-1 for β-glucosidase; 42 and 52 μg N-NH4 g-1h-1 for urease respectively for fertilized soil and reference. In amended soil (C and P), β-glucosidase was positively correlated with phosphatase activity ($P < 0.001$) and those enzyme activities with organic C ($P < 0.01$), which explain that the inclusion of organic manures furnishes substrates for the both enzymes and improves microbial expansion. The raised organic carbon noted in the fertilized soil suggests the great feedback of this soil to the amendment by the organic manure, than that of the reference soil.

This is in conformity with numerous works that have suggested that amending soils by organic matter increases the enzyme activities (Bol et al., 2003). Augmentation in activities is perhaps depending to the metabolic ability of the microbial communities and their capacity to use the provide substrates (Falih and Wainwright, 1996). Eivazi and Bayan (1996) also spotted that, phosphatase, β-glucosidase, arylsulfatase and urease activities were effectively correlated with the total microbial biomass.

In this work we see that the urease activities were decreased in amended soil. This probably resulted to the inhibition of urease produced by the nitrogen that fabricated under conversation of NH_4^+ (Bandick and Dick, 1999). Great microbial activity in the fertilized soil creates a big request for nitrogen and, thus, higher conversion of NH_4^+, which may induce a reduction in the urease activity.

Table 3 The enzyme activities measured.

Enzymes		Phosphatase (μg pNP g^{-1})	β-Glucosidase (μg pNP g^{-1})	Urease (μg N-NH g^{-1})
Farmers	R	88.5	41.4	52
	CM1	447.0	92.0	31.6
	CM2	719.4	121.1	36.2
	CM3	483.2	89.5	32.8
	PM1	402.5	62.0	40.1
	PM2	413.6	76.3	44.0
	PM3	389.0	75.0	42.5

3.3. Microbial diversity

DGGE showed the existence of 18 bands in soil amended by poultry manure and 25 bands in soil amended by cow manure, corresponding to bacteria, while there were only 7 bands in the soil reference (Fig. 1).

To better discover the variance in the bacterial communities of the soil with cow or poultry manure, the structural variety of the bacterial communities was also inspected by the Shannon (H') and Simpson (D) diversity index (Table 4).

The results show that Shannon index was significantly higher in fertilized soil than reference (R) and particularly higher for soil amended by cow manure. Contrariwise, the Simpson (D) diversity index shows a decrease in the amended soils than the reference one.

The Shannon–Weaver diversity index, H′, and microbial diversity are absolutely correlating. However, the Simpson index, D, negatively correlated with the microbial diversity. In this study, the Simpson index, D, and the Shannon–Weaver diversity index, H′, showed converse trends, which indicated that the results of D were coherent with those of H′.

Poultry and cow manures hold a huge density of various microorganisms that have major roles in the ecological conversion and transport of the C, N, P and other nutrients that are presented in the dejected or litter material.

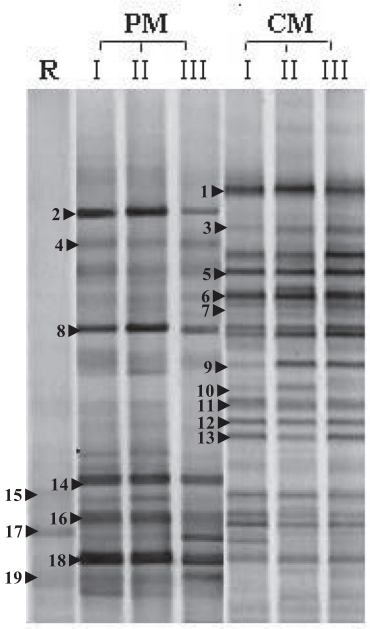

Figure 1 DGGE banding profiles of 16S rRNA genes of different soil samples (R: reference; PM: soils amended by poultry manure; CM: soils amended by cow manure).

Table 4 Shannon index of general diversity (H') and Simpson index of dominance (D).

	R	CM1	CM2	CM3	PM1	PM2	PM3
H′	1.08	2.114	2.088	2.109	1.255	1.223	1.241
D	0.198	0.103	0.97	0.108	0.189	0.198	0.191

The manure amendment significantly augments the bacterial diversity (Table 4). The fertilization increased the Shannon–Weaver diversity index of bacteria, and augmented soil microbial group richness, i.e., the number of various bands existed in samples.

3.4. DNA sequencing and phylogenetic analysis

Partial sequencing of 31 bands (500–550 bases per band) excised from prominent DGGE bands was realized. Sequences were submitted to the NCBI BLAST search engine and a putative phylogenetic position was assigned to each sequence based on the results (Table 5).

The sequences consisted of 19 dominant bands excised from DGGE profile of soils amended by the cow manure and poultry manure, where 10 dominant bands excised from DGGE profile of soil amended by CM, and 6 dominant bands retrenched from DGGE profile of that amended by PM. Finally, 3 dominant bands were also identified from the DGGE gel of reference soil.

In this work, variation of amending had a real impact on the soil microbial community. Differences at a fine taxonomic level were demonstrated by the PCR–DGGE analysis of bacterial rRNA gene sequences in soil, which showed that CM had a high effect than PM on bacteria species. Some precise strains were detected mainly in amended soils due to the propitious conditions for the multiplication and activity of special microbial ribotypes (Beauregard et al., 2010).

Additionally, many microorganisms could be multiplied, and others could be removed. Variances in the bacterial diversity in our work could result in shifts the quality and functionality of soils. In general, in this study organic manure fertilization increased microbial taxa, which the order Bacillales and related genera were mainly detected in the soil fertilized by cow manure and the members of the order Clostridiales and Pseudomonadales were mainly detected in the soil with poultry manure. Then, in the gel of sample obtained from soil amended by cow manure (CM), all the excised bands revealed a resemblance to the phylum Firmicute (*Bacillus*, Exiguobacterium sp... etc) and Bacteroidetes (Flavobacterium spp). However, in the gel of sample obtained from soil amended by poultry manure (PM) the excised bands revealed a resemblance to the phyla β-Proteobacteria (Pseudomonas sp.) and Firmicutes (Staphylococcus and Clostridium). The order Bacillales has been known to be very disseminated in different organic manures such as cow, cattle, pig and poultry (Green et al., 2004; Lovanh et al., 2007).

Furthermore, the greater parts of pathogenic strains were found in the soil amended by poultry manure; such as *Staphylococcus simulans*, *Staphylococcus sciuri*, and *Pseudomonas aeruginosa*. These species and others pathogenic strains were been found through previous work of poultry manure characterization (Lovanh et al., 2007; Wilkinson et al., 2011). The natural habitats of most the enteric pathogens are gastrointestinal tracts of animals. Whenever animal rejections are used into the farming activity, the antagonistic impact of native soil microbial community and the drastic conditions of soil may modify the longevity of pathogens.

3.5. Cluster analysis of DGGE fingerprints

The DGGE band position, number, and density had strong resemblance between the rehearsals for all soil samples, which recommended that the rehearsal DNA characterization was excellent enough to discriminate the difference between the taxa communities among the analyzed soils in this work. DGGE profile discovered that majority of DGGE bands were very analogous, which deduct that the ribotype strains and those bands were permanent and popularly presented in cultivated soil (Fig. 1). The likeness dendogram takes on the Dice

Table 5 Partial sequence analysis of bacterial 16S rDNA genes recovered from soil under application of cow and poultry manure.

Band number	Name	Accession no.	% Similarity	Phylogeny	Order	Samples assigned soil
1	*Chryseobacterium* sp. *CPW406*	AJ457206	98	Bacteroidetes	Flavobacteriales	CM
2	*Staphylococcus Cohnii*	AB 009936	99	Firmicutes	Bacillales	CM & PM
3	*Bacillus cereus*	AJ577283	100	Firmicutes	Bacillales	CM
4	*Chryseobacterium scophthalmum*	AJ271009	96	Bacteroidete	Flavobacteriales	CM & PM
5	*Bacteroidetes* sp. *RW262*	AF493694	97	Bacteroidetes	Flavobacteriales	CM
6	Uncultured Firmicutes bacterium clone	EU297149.1	97	Firmicutes	-	CM
7	*Exiguobacterium* sp.	AF275715	99	Firmicutes	Bacillales	CM
8	*Clostridium* sp.	AJ318890	98	Firmicutes	Clostridiales	PM
9	*Listeria monocytogenes*	KJ765661	98	Firmicutes	Bacillales	CM
10	*Lactobacillus aviarius*	AB 001836	99	Firmicutes	Lactobacillales	CM
11	*Pseudomonas* sp. *BBMB*	KF965279	98	γ-Proteobacteria	Pseudomonadales	PM
12	*Ruminococcus obeum*	X85101	99	Firmicutes	Clostridiales	PM
13	*Staphylococcus simulans*	D83373	99	Firmicutes	Bacillales	PM
14	*Pseudomonas aeruginosa*	FJ665501	99	γ-Proteobacteria	Pseudomonadales	PM
15	*Uncultured Sphingobacteriaceae*	AJ252602	98	Bacteroidetes	Sphingobacteriales	R & CM
16	*Staphylococcus sciuri*	EU095646	99	Firmicutes	Bacillales	PM
17	Uncultured Campylobacter sp.	KR848846	98	γ-Proteobacteria	Campylobacterales	R & CM
18	*Eubacterium desmolans*	L34618	99	Firmicutes	Clostridiales	CM & PM
19	Uncultured Arthrobacter sp. clone	VE34	97	Actinobacteria	Micrococcales	R

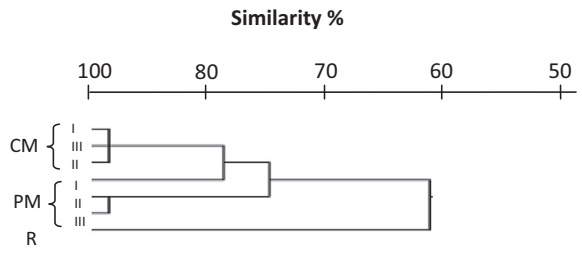

Figure 2 Cluster analysis of DGGE banding profiles.

coefficient (3% error) for the presence and absence of bands demonstrate a similarity of 76% between all analysis samples. These demonstrate that there is a pointed dissimilarity between the microbial communities in the two soils.

The noted discordance of banding profiles was might due to several bands or band density. Cluster description of DGGE banding profils of bacterial communities showed that the cluster was subdivided into three major groups (Fig. 2). Group I composed of all soil samples amended by cow manure, and group II contained the soil samples amended by poultry manure as compared to reference (group III).

Soil fertility may affect the activities and diversity of the soil biodiversity in different action (Liu et al., 2008). It could influence microbial development and competitiveness due to the differences of ability between microorganisms to treat the diverse nutrient forms found in soil. Especially, the accessibility of nutrients such as C, N, and P can affect soil microbial development and activity (Broeckling et al., 2008).

However, the function, structure, of soil population germs were affected in long-term fertilization experiments (Cinnadurai et al., 2013), due to the hard favors of bacterial residues accumulation from these fertilizers (Murugan and Kumar, 2013).

An augmentation in the quantity or biodiversity of soil is generally considered as advantage. Conversely, an augmentation in the microbial population inducts an increase of nutrient immobilization, and augmentation in soil organic matter can amplify populations of pathogen germs as like as parasitic.

Preservation of soil biodiversity is decisive to soil health and quality, as numerous microorganisms are responsible for essential biochemical functions in soil. Microbial diversity composition and the necessary time to back to a stable situation after the application of diverse perturbation or stress may be a helpful sign of potential perturbation of soils supervising. Though many germs utilize special metabolic actions inside the native community of soil, huge amount of environment microbiotra could interfere with the survival and expansion of natural manure. Fully understanding the levels and prevalence of human pathogens in poultry manure is important for adopting policy for surveying contagion on culturing area.

4. Conclusions

This work proves that the type of fertilization may considerably modify the organization of soil microbial communities mainly by varying the soil chemical characteristics and its fertility conditions.

The evaluation of bacterial community using DGGE technique showed that the addition of organic manure caused a modification of soil bacterial community structure. This community structure change is clearer if we take into account the band's intensity and not only his presence:absence. This may be explained by a concurrence effect between the autochthones' community of soil and the added ones. The reason for this hypothesis is that the diversity was usually high in the samples that received manure, however some bacterial strains were not in the control soil (R) but appeared only in the amended soil (PM and CM). These strains could have a huge adaptation capacity by their resistance to the telluric strains.

The study showed also the presence of a variety of human pathogens in soil amended by poultry manure (PM) belonging to the members of the order Clostridiales and Pseudomonadales, that could menace humans who consume the infected food or water.

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