UNIVERSIDADE FEDERAL DO PARANÁ

ROCÍO DEL PILAR CUASPA ROPAÍN

BIODIVERSIDADE MICROBIANA E PROSPECÇÃO METAGENÔMICA EM AMOSTRAS DE TRATAMENTOS DE RESÍDUOS PECUÁRIOS

CURITIBA

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BIODIVERSIDADE MICROBIANA E PROSPECÇÃO METAGENÔMICA EM AMOSTRAS DE TRATAMENTOS DE RESÍDUOS PECUÁRIOS

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Orientadora: Prof.ª Dr.ª Leda Satie Chubatsu

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Curitiba, 31 de Julho de 2018.

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Gabriel García Márquez

RESUMO

Dada a dimensão da indústria pecuária de aves e suínos no Brasil, é fundamental destinar os resíduos produzidos pela mesma de forma adequada. Uma das formas de tratamento é através da compostagem do material. De forma tradicional em pilha ou em rotoacelerador, a compostagem é um processo realizado principalmente por microrganismos transformando a matéria orgânica em composto nutriente útil como adubo. Para este trabalho, foram analisadas amostras de compostagem de 5 granjas comerciais, através da extração e sequenciamento do DNA metagenômico para análise de biodiversidade por amplificação do gene 16S rDNA ou do DNA total por sequenciamento "shotgun", ambos utilizando a plataforma Illumina MiSeq. Constatou-se que o filo Firmicutes foi o mais abundante em todas as amostras, seguido de Actinobacteria, Proteobacteria e Bacteroidetes. O gênero *Nasocomiicoccus* foi encontrado associado às amostras que continham resíduos de frango enquanto o gênero *Bacillus* às amostras de resíduos de suínos. Quando comparadas as amostras tratadas em pilha tradicional e em rotoacelerador não foi encontrada diferença significativa na estrutura microbiológica, concluindo que tratando os resíduos em equipamento acelerado, uma vez que o processo de compostagem acontece de forma mais rápida e limpa, trazendo vantagens para as granjas comerciais dessa indústria crescente.

Outra parte deste trabalho envolveu a identificação de uma nova enzima com potencial aplicação biotecnológica através do sequenciamento de bibliotecas metagenômicas construídas a partir de amostras de uma lagoa de descarte de indústria agropecuária. Nesta etapa a lipase Lip720E foi identificada por análise *in silico*, expressa em *Escherichia coli* e testada para atividade lipase com diferentes substratos de ácidos graxos. Esta análise permitiu comparar a estratégia de identificação por análise baseada na função de outra lipase apresentada na mesma biblioteca.

Palavras-chave: compostagem, biodiversidade bacteriana, lipases bacterianas, metagenômica.

ABSTRACT

Due to the relevance of the poultry and swine husbandry industry in Brazil, proper waste destination gains importance. One of the possible treatments is composting, and it can be carried out using a traditional static pile or a roto-accelerator. This process is performed mainly by microorganisms transforming organic matter in nutrient compost for crops. In this work, composting samples of 5 commercial farms were analyzed by extracting and sequencing metagenomic DNA using the 16S rRNA biodiversity approach or the whole DNA sequencing by "shotgun", both using the Illumina MiSeq platform. Phylum Firmicutes was found as the most abundant one in all samples, followed by Actinobacteria, Proteobacteria and Bacteroidetes. Genus *Nasocomiicoccus* was found associated with poultry waste samples and *Bacillus* genus with swine waste samples. When compared samples treated in traditional pile with those from roto-accelerator no difference in microbial structure was found, concluding that treatment in accelerated equipment may represent an advantage for a faster and cleaner composting process in a growing number of commercial farms.

This work also included the identification of a new enzyme with potential biotechnological application through the sequencing of metagenomic libraries from samples of a waste lagoon of husbandry industry. In this section the lipase Lip720 was identified by *in silico* analyses, expressed in *Escherichia coli* and tested for lipase activity with different substrates for fatty acids. This analysis allowed comparison of identification strategy function based with another lipase from the same library.

Key-words: composting, bacterial biodiversity, bacterial lipase, metagenomics.

RESUMEN

Dada la dimensión de la industria pecuaria de aves y cerdos en Brasil, es fundamental dar un destino adecuado a sus residuos producidos. Una de las formas de tratamiento es a través del compostaje, que puede ser de forma tradicional en pila estática o en rotoacelerador. Este proceso es realizado principalmente por microorganismos transformando la materia orgánica en compost útil para cultivos. En este trabajo, fueron analizadas muestras de compostaje de 5 granjas comerciales a través de la extracción y secuenciamiento de ADN metagenómico para análise de biodiversidad por amplificación del gen 16S ADNr o del ADN total por secuenciamiento "shotgun", ambos utilizando la plataforma Illumina MiSeq. Se encontró que el phylum Firmicutes fue el más abundante en todas las muestras, seguido de Actinobacteria, Proteobacteria y Bacteroidetes. Se encontró que el género *Nasocomiicoccus* estaba asociado a muestras de residuos de aves, mientras que el género *Bacillus* a muestras de residuos porcinos. Al comparar muestras tratadas en pila tradicional y en rotoacelerador, no se encontró diferencia en la composición microbiana, permitiendo concluir que el tratamiento en equipamento acelerado tiene más ventajas para el número creciente de granjas comerciales al ser un proceso más rápido y limpio.

Otra parte de este trabajo implicó la identificación de una nueva enzima con potencial aplicación biotecnológica a través del secuenciamiento de bibliotecas metagenómicas construidas a partir de muestras de una laguna de descartes de industria agropecuaria. En esta etapa se identificó la lipasa Lip720E por análisis *in silico*, se expresó en *Escherichia coli* y se analizó para actividad lipasa con diferentes sustratos de ácidos grasos. Este análisis permitió comprar la estrategia de identificación por análisis basado en la función de otra lipasa contenida en la misma biblioteca.

Palabras claves: compostaje, biodiversidad bacteriana, lipasas bacterianas, metagenómica.

SUMÁRIO

INTRODUÇÃO

A criação de animais em sistemas confinados acarreta grande acúmulo de resíduos. No caso da avicultura e suinocultura para produção de carnes, ovos, laticínios e outros, estas indústrias pecuárias tem ganhado um lugar importante na economia brasileira, sendo uma das maiores do mundo. O Brasil é o segundo produtor mundial de carne de frango e o quarto de carne suína, essa posição é resultado de um processo de produção eficiente e altamente regulado (ABPA, 2017). No entanto, essa produção gera uma grande quantidade de resíduos potencialmente contaminantes, só de rebanho suíno nacional a quantidade estimada é de 300.000.000 milhões de m^3 (Ferreira, 2014).

Esses resíduos pecuários incluem dejetos como fezes e urina, pelos ou penas, restos de comida, bebida, cama de criação e carcaças de animais que não conseguem chegar até o fim do processo produtivo. Além disso, o próprio processamento dos animais na indústria também gera resíduos biológicos e químicos.

Antigamente os resíduos pecuários eram despejados para o meio ambiente sem tratamento nenhum já que esses dejetos não representavam problema ao meio ambiente, entretanto novas legislações para avicultura e suinocultura intensivas obrigam tratamento e despejo adequado desses resíduos biológicos e químicos. Entre as diferentes formas de tratamento e/ou utilização dos resíduos incluem a compostagem e o descarte em lagoas isoladas. Estes são usados como meios de decomposição da matéria orgânica, ciclagem de nutrientes e elementos químicos, redução da carga microbiana e fermentação da biomassa. No caso do processo de compostagem, como resultado tem-se a produção de um composto orgânico útil para adubação de solos e fertilização para lavouras, gerando um valor agregado aos resíduos da indústria.

Assim como muitos processos biogeológicos, os processos de tratamento de resíduos da indústria pecuária são realizados em grande parte pela atividade de microrganismos. Como descreveu Handelsman (1998) ao definir os metagenomas como o estudo dos microrganismos de um ambiente dado, estes seres vivos se encontram presentes em todos os ambientes possíveis do planeta e possuem vias metabólicas diversas cumprindo as mais variadas funções bioquímicas. Segundo o Comitê de Metagenômica do Conselho de Pesquisa Nacional dos Estados Unidos, a microbiologia se encontra em um momento sem precedentes para revolucionar o entendimento do mundo vivo (Committee on Metagenomics: Challenges and Functional Applications, 2007).

A biodiversidade microbiana nos ambientes de compostagem ou lagoas de dejetos é pouco conhecida e os poucos estudos encontrados foram realizados com técnicas tradicionais de microbiologia dependentes de cultivo e são limitadas às condições laboratoriais. Como em outros ambientes, a grande maioria de microrganismos não cultiváveis permanece desconhecida (Martins *et al.*, 2013; Delgado-Baquerizo *et al.*, 2018) e as novas tecnologias de sequenciamento oferecem uma grande probabilidade na identificação de um amplo número de grupos taxonômicos bacterianos em menor tempo a cada vez um menor custo, possibilitando o encontro de novos grupos taxonômicos e permitindo compreender suas funções.

De outra parte, o aproveitamento das variadas funções metabólicas microbianas através de ferramentas biotecnológicas oferece um potencial recurso para a bioprospecção e aplicação industrial. Uma das aplicações biotecnológicas com amplos estudos e potencial comercial é a das enzimas de degradação de lipídeos, enzimas do grupo das hidrolases que incluem as lipases e esterases (Böttcher et al., 2010; Lee e Lee, 2013). Estas enzimas possuem características interessantes como o não requerimento de cofatores, destacada estabilidade em solventes orgânicos, amplo reconhecimento de substratos, podendo ainda apresentar estereoseletividade e seletividade posicional (Lee et al., 2004).

O objetivo deste trabalho foi utilizar ferramentas metagenômicas para determinar a riqueza, equabilidade e composição microbianas em amostras de tratamentos de resíduos de avicultura e suinocultura, e realizar a bioprospecção na busca de novas enzimas.

Nesse projeto utilizamos técnicas da metagenômica para análise do material genético obtido diretamente do ambiente sem cultivo celular prévio e/ou identificação individual de amostras/indivíduos. O sequenciamento de DNA foi realizado utilizando metodologia de nova geração (plataforma Illumina MiSeq) para determinar a biodiversidade bacteriana de amostras de compostagem. Através desse trabalho foram possíveis comparações entre as compostagens de resíduos de criação de aves ou suínos, processados em pilha estática ou em rotoacelerador.

Na segunda parte deste projeto, foi caracterizada uma enzima com atividade lipolítica identificada a partir da prospecção *in silico* de uma biblioteca metagenômica (Glogauer et al., 2011). Esta parte do trabalho permitiu a comparação entre uma triagem funcional e *in silico* na prospecção por lipases.

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CAPÍTULO I

Biodiversidade bacteriana da compostagem de resíduos de avicultura e suinocultura

Bacterial diversity of waste composting from poultry and swine farming

Rocío Cuaspa, Leda S. Chubatsu.

ABSTRACT

Microorganisms are the main contributing factors responsible for organic matter degradation during composting. Wastes from poultry and swine production, such as carcasses and litter, are being treated by traditional pile or roto-accelerated composting process to produce plant fertilizers. In this work, Illumina DNA-sequencing of the 16S rRNA gene was used to determine the bacterial community structure and functional potential of animal farm composting using a traditional static pile or a roto-accelerator composting process of avian or swine carcasses. Analyses indicated that Firmicutes was the main phylum present in all samples, followed by Actinobacteria, Proteobacteria, and Bacteriodetes. PCA analysis of the relative number of sequences showed a difference between the composts according to the animal source, avian or swine, but no clear difference was found between the two types of composting treatments, static pile or roto-accelerated. To statistically address differential abundance, The *Nasocomiicoccus* genus was found to feature avian source samples, while *Bacillus* genus features in swine source samples, suggesting that these two groups could be used as markers for animal waste composting. Shotgun sequencing of total metagenomic DNA indicated similar functional profile between the animal source and between the type of treatment. This is the first study of composting wastes from commercial farms of poultry and swine farming, addressing a traditional pile protocol and an equipment to decrease the incubation period for composting.

Key words: animal husbandry, animal farm waste composting, bacterial community structure.

1. INTRODUCTION

Brazil is the world's second producer of avian meat and the forth of pork meat having one of the largest herds in the world. Although this livestock industry has been highly regulated throughout the setting parameters for an efficient production process, more than one million tons of dead animals are produced per year as part of regular farming breeding process (ABPA, 2017). According to Nicoloso *et al.* (2017), the disposal of dead animals within the limits of the rural establishment involves the removal of carcasses from the breeding place, eventual temporary storage, transport and treatment system, and composting is the most widely used method for totally decompose carcasses by the action of microorganisms. These composts are mainly used as plant fertilizer.

In addition to poultry and swine carcasses, farming wastes are also composed of litter, which has served for bedding the animals. This is made of a mixture of vegetal substrates such as sawdust, wood shavings, corncob, rice straw, coffee bark and others. After usage, this litter contains urine, feces, feathers or hair and remnants of food and drink. The used animal bedding and the carcasses of animals that eventually die during breeding are conventionally processed by traditional composting consisting of a static pile made up with a layer of vegetal matter, a layer of animal carcasses, and then another layer of vegetal matter. These successive layers are repeated until one last layer of vegetal material covers up the whole pile that should be assembled in windrows. Dimension, time, and composition may vary according to each case following technical parameters in the field.

Composting refers to the biodegradation process occurring spontaneously in nature and reproduced on human conditions, starting from a mixture of organic substrates in solid state, humidity and conditions of both aerophilia and microaerophilia. The exothermic process of highly metabolically

active microorganisms produces energy in the form of heat, resulting in an increase of the temperature and degradation of polymers and organic compounds to a final product of a stabilized organic matter beneficial to plant growth (Insam and de Bertoldi, 2007).

It is generally accepted that composting consists of mesophilic phase, thermophilic phase and maturation phase (de Gannes *et al.*, 2013; Zhang *et al.*, 2016). Some authors add a cooling phase or second mesophilic phase (Insam and de Bertoldi, 2007). In a well-managed process, about 50% of the biodegradable organic matter is converted into CO_2, H_2O, mineral salts, and energy. The factors which affect this conversion are: system of composting, duration of the process, aeration system, chemical and physical composition of the organic matter, particle size, carbon/nitrogen ratio, and temperature. These parameters have shown to be key for composting optimization since they determine conditions for microbial development and organic matter degradation (Bernal *et al.*, 2009).

Microorganisms are present in all biomes on Earth. In soils, sediments, water bodies and all natural conditions, organic matter transformation and nutrients cycling is carried out by microorganisms as the main contributing factor (Handelsman, 2004). Current knowledge of microbial community is largely based on traditional methods of isolation and culture by direct microscopic observation and plating procedures. During the composting process, microorganisms are induced into high metabolic activities growing at high densities at constant change of environmental conditions, favoring exponential growth of certain kind of microorganisms, and inducing stationary phases of others. However, only a minor fraction can be cultivated with conventional laboratory equipment and procedures, resulting in the known "the great plate count anomaly" that refers to the discrepancy between the viable plate count and total direct microscopic count of bacteria (Schloss and Handelsman, 2003; Staley and Konopka, 1985). Several culture methods have been developed since Lane *et al.* (1985) proposed direct DNA cloning from

environmental samples. Current consensus on accessing complexity on microbiota information resides on next generation high-throughput DNA sequencing potential, which has become increasingly faster and less expensive (Boughner and Singh, 2016).

Recently, in order to increase the efficiency of composting, an equipment was developed. The Roto-Accelerator of Composting (AgroBona Indústria de Equipamentos, PR, Brazil) allows controlling the variables of the composting process promoting environmental and sanitary safety. In this treatment, whole carcasses and other organic residues are placed inside the drum of the equipment that remains sealed, with constant mixing. Temperature and moisture are constantly monitored, and carbon and nitrogen sources are adjusted according to user parameters, generating more homogeneous and stable compost in less than half of the time from traditional pile composing. Moreover, benefits of this technology include the disposal of carcasses with less environmental impact, safer handling for the farm worker, and the production of a higher quality plant fertilizer.

In characterizing composting microbiome community, some authors tested either sheep or cow manure with different vegetal substrates, rice straw, coffee hulls, and sugar cane bagasse (de Gannes *et al.*, 2013), cattle tissues and manure with straw (Tkachuk *et al.*, 2014), animal and vegetal wastes from a zoo (Martins *et al.*, 2013) and soil (Delgado-Baquerizo *et al.*, 2018). However, no current reports using high- throughput DNA sequencing applied to avian or swine waste and carcasses from husbandry composting has been published. Therefore, information about bacterial community composition during traditional or accelerated composting remains scarce. This type of study of microbial community structure analysis could result in improvement of composting facilities by adjusting appropriate conditions for an efficient microbiological process.

In this study, we analyzed the bacterial biodiversity of composting from poultry farming and a swine farming comparing traditional pile composting or

accelerated process using a roto-accelerator equipment. We hypothesize that these two types of treatment are comparable in terms of microbiome composition, regardless the animal source of the waste. To test this, we addressed two main approaches: taxonomical structure and functional potential analyses. To our knowledge, this is the first report of using high-throughput DNA sequencing applied to avian or swine waste and carcasses from husbandry composting.

2. MATERIAL AND METHODS

2.1 Samples

For this study, samples of organic matter at different stages of the composting process were used from poultry or swine farms. Composting of poultry carcasses included animal bedding of mixed vegetal litter and composting of swine carcasses that do not use litter, included pine shavings. Protocols considered traditional or roto-accelerator equipment.

Composting samples were obtained from 5 commercial farms located in Toledo and Maripá, Paraná State of Brazil, on December 1st, 2014. Samples were provided by BRF Company. Farms composted animal waste either from avian farming (here indicated as Av.) or swine farming (indicated as Sw.); composting protocols were performed either in a static pile of composting (indicated as Com.), or in a roto-accelerator (indicated as Rot.); also, in the case of swine, a composting from adult animal (indicated as Sw.Com.t) or young animal (indicated as Sw.Com.c) were analyzed. Geographical locations of sample collections were: Av.Com: 24º 30' 44,1" S 53º 43' 52,6" W; Av.Rot: 24º 28' 32,8" S 53º 48' 43,4" W; Sw.Rot: 24º 26' 29,6" S 53º 48' 00,7" W; Sw.Com.t: 24º 29' 10,0" S 53º 48' 37,1" W; Sw.Com.c: 24º 33' 04,8" S 53º 52' 15,1" W.

Composting materials were homogenized, collected and stored in 50 mL

plastic capped tubes, and maintained on ice until arriving to the lab and then kept frozen at -45 °C.

Samples from 4 biological replicates were mixed to promote homogenization of starting material, and 250 µg was used to extract DNA from the microbial community with 3 technical replicates using *Power Fecal Isolation Kit* (MOBIO, Carlsbad CA) according to manufacture instructions. Metagenomic DNA quality was evaluated by electrophoreses and quantified with Qubit (Invitrogen).

2.2. DNA Sequencing

Metagenomic DNA from each sample was used as template to amplify the V4 region of 16S rRNA gene (Caporaso *et al.*, 2012; Caporaso *et al.*, 2011). Amplification was carried out using 10 ng of metagenomic DNA, KlenTaq Mix polymerase (Sigma-Aldrich) and 10 mM of primers 515F (AATGATACGGCGACCACCGAGATCTACACTATGGTAATTGTGTGCCAGCMGCCGCGGTA) and 806R (CAAGCAGAAGACGGCATACGAGATAGTCAGTCAGCC-GGACTACHVGGGTWTCTAAT). Reaction occurred with initial denaturation temperature of 94 °C for 3 minutes, followed by 18 cycles of denaturation at 94 °C for 45 seconds, annealing at 50 °C for 30 seconds, and extension at 68°C for 60 seconds, with final extension at 72 °C for 10 minutes. Amplified DNA products of 400 bp were precipitated and barcoded with Nextera kit and sequenced using Illumina MiSeq2000 platform according to manufacturer´s instructions.

Also, from two random samples global sequencing of total DNA was performed in Illumina MiSeq2000 platform according to manufacturer´s instructions by shotgun strategy.

2.3 DNA Sequences analyses

Sequences of F1 strands were processed, demultiplexed and filtered using Qiime v.1.9.1 (Caporaso *et al.*, 2010). Reads shorter than 240 pb were eliminated, and from those selected, quality acceptance was 0.5. Sequences were treated with Uparse (Edgar, 2013) and denoised with Usearch9 (Quince *et al.*, 2011). Chimeras were removed, and taxonomy was assigned through Operational Taxonomic Units (OTUs) that were generated at 97% of identity using Silva database release 128 (Yilmaz *et al.*, 2014). Core analysis of biodiversity was performed with 1,114,635 reads in Qiime and normalization with 7860 reads. The OTUS visualization of alpha and beta diversity analyses, and the multivariated analyses of principal components (PCA) with alpha 0.5 were performed in Emperor (Vazquez-Baeza *et al.*, 2013). Also, alpha diversity analysis at level of genera groups were calculated in R (R Core Team, 2017) using BiodiversityR version 2.9-1 package (Kindt and Coe, 2005) including richness, abundance, Shannon and Simpson indexes. The Rényi profile that generates a visual comparison of biodiversity indexes was generated in R using Vegan version 2.4-6 package (Oksanen *et al.*, 2018). Statistical analysis of metagenomic profile was done in STAMP v.2.1.3 (Parks *et al.*, 2014) with extended error bar was used for two genera groups, indicating the genera with p-value <0.05, according to two sided t-test with confidence interval of 0.95 and t-test inverted; multiple test correction was done with Bonferroni.

From global sequencing of total DNA of two random samples, the set of unassembled reads was submitted for functional annotation to Metagenomics Rapid Annotation (MG-RAST) pipeline (Meyer *et al.*, 2008). The functional profile was analyzed with SEED database subsystem level 1 (Overbeek *et al.*, 2005) that provided more number of categories and presented the highest number of annotated sequences compared to the other databases provided by the platform.

2.4 Physico-chemical measurements

Carbon and nitrogen content of each sample was determined in

Department of Soils and Agriculture Engineering at the Federal University of Paraná (Curitiba, Brazil). Briefly, each composting sample was homogenized, dried in oven at 80 °C for 24-28 hours to completely eliminate moisture, macerated to fine powder and sifted through a fine sieve. A fraction of thirty five mg of powdered sample was encapsulated in a tin foil, and submitted to combustion with He/O_2 atmosphere. Detection and quantification of total content of organic matter was performed in an elemental analyzer (Elementar, Germany).

Sample moisture was determined by weighting 3 g of homogenized samples before and after drying in oven at 60 °C for 9 days. For pH determination, 20 mL of fresh homogenized composting samples were mixed with equal volume of $CaCl_2$ 0.01M and the pH measured with a pHmeter.

3. RESULTS

The composting samples analyzed in this work were obtained from waste composting in different farms regardless animal source or treatment type. The physical-chemical parameters of the composting material are in accordance with isolated points of the composting process and constitute non comparable samples. The Carbon/Nitrogen ratio, moisture percentage and pH are characteristic of different phases of composting process. Sample parameters are summarized in **Table 1**.

Table 1. Samples characteristics. Samples correspond to isolated points of husbandry waste composting; they are not comparable and were analyzed as described in Material and Methods.

Abbreviation	Type of treatment	Length of treatment* (days)	Temperature at sampling (°C)	C/N**	pH	Moisture (%)
Av.Com.	Avian Composting	13	41.2	10.2	8.7	43.7
Av.Rot.	Avian Rotoaccelarator	30	35.8	9.3	7.2	14.5
Sw.Rot.	Swine Rotoacelerator	110	53.6	7.1	8.1	26.2
Sw.Com.t	Adult Swine Composting	60	39.6	8.6	7.5	57.4
Sw.Com.c	Young Swine Composting	130	35.8	23.7	7.6	13.7

*Length of treatment indicates composting period
**Carbon/Nitrogen ratio

For each sample 3 technical replicates were processed for DNA extraction resulting in 15 samples, assessed by visualization in agarose gel electrophoreses (**Figure 1**). These 15 extracted DNA samples were submitted to 16S rRNA amplification of V4 region as indicated in material and methods, and products were sequenced and the number of reads obtained from each sample are indicated in **Table 2**. Samples with low number of reads were submitted to a new sequencing run and reads were combined to achieve a minimum number of reads for normalization at OTU determination. After quality filtering, 91.2% of reads were obtained with a total of 1,114,635 reads, and richness indexes were calculated starting from 7,860 reads as the even sample depth (**Table 2**).

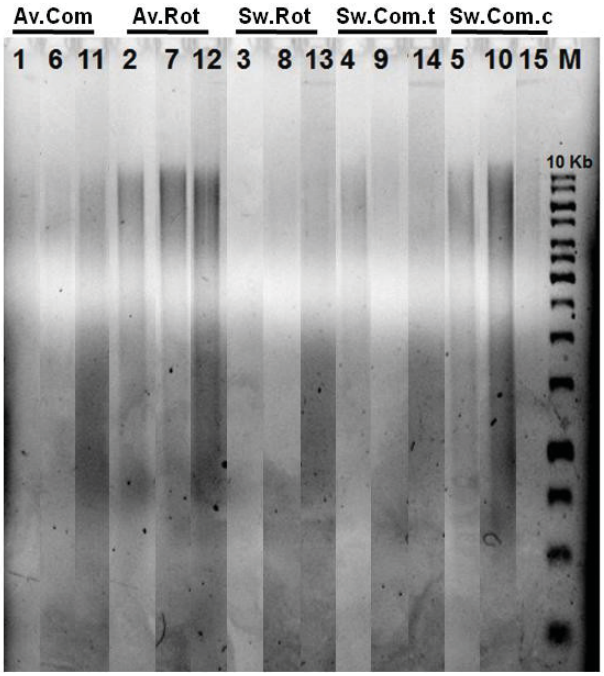


Figure 1. Electrophoresis in 1% agarose gel of purified metagenomic DNA. Samples are grouped together by replicates: Av.Com (Avian Composting); Av.Rot (Avian Rotoaccelerator); Sw.Rot (Swine Rotoaccelerator); Sw.Com.t (Swine Composting adults); Sw.Com.c (Swine Composting young individuals). Numbers indicate each replicate. **M** indicates the molecular weight markers (1 Kb ladder -Fermentas). Ethidium bromide was used for staining.

Table 2. DNA sequencing data. Each treatment gathers three technical replicates. The column of Sample indicates the number of the technical replicate. The column of Reads indicates the number of sequenced reads per sample.

Treatment	Sample	Reads	Sample	Reads	Sample	Reads	TOTAL
Av.Com	1	68784	6	100458	11*	71247	240489
Av.Rot	2	107965	7	38849	12	132902	279716
Sw.Rot	3	128317	8*	10805	13	27500	166622
Sw.Com.t	4	63143	9*+	7861	14	139472	210476
Sw.Com.c	5	57327	10	104499	15	55506	217332
Total							**1114635**

*number of reads after 2 gathered runs (combined)
+ cutline for OTU determination

The number of OTUs was determined showing a variation per treatment from 266 to 1191 on average (**Table 3**). When compared diversity of

treatments by comparing samples from pile composting to those from rotoaccelerator, alpha richness analyses indicated higher average values from pile composting, including number of OTUs, richness, and estimator indexes of Chao, PD whole tree, Shannon and Simpson (**Table 3**). Moreover, Rényi profile (**Figure S1**) that allows comparison among indexes, indicated a dominance pattern of all samples, confirmed a higher richness of pile composting samples and revealed that some replicates with intersecting profiles, as explained by Tóthmérész (1995), are non-comparable communities.

Table 3. Alpha diversity indexes per treatment. From 16S rRNA amplicon sequence dataset of composting samples are shown the number of OTUs, richness indices of Chao 1, Phylogeny Distance (PD) whole tree, values of richness and abundance, Shannon index and Simpson index. Replicates per treatment are shown in samples and average values were calculated. Treatments: Av.Com (avian composting), Av.Rot (avian rotoaccelerator), Sw.Rot (swine rotoaccelerator), Sw.Com.t (swine composting of adults carcasses), and Sw.Com.c (swine composting of young carcasses).

Treatment	Sample	# OTUs	Chao 1	PD whole tree	richness	abundance	Shannon	Simpson
Av.Com.	1	449	1257	26.47	357	7426	3.82	0.94
	6	428	1243	26.12	316	6227	4.01	0.96
	11	723	1920	40.53	479	5955	4.40	0.97
	Average	**533**	**1473**	**31.04**	**384**	**6536**	**4.08**	**0.96**
Av.Rot.	2	201	603	15.59	158	7711	1.54	0.52
	7	203	629	14.57	163	7708	1.63	0.53
	12	395	1137	26.49	292	7333	2.40	0.68
	Average	**266**	**790**	**18.88**	**204**	**7584**	**1.86**	**0.58**
Sw.Rot.	3	143	922	11.66	96	6914	1.61	0.63
	8	262	609	15.84	217	7033	2.92	0.86
	13	641	1273	35.85	485	6872	4.13	0.95
	Average	**349**	**935**	**21.12**	**266**	**6940**	**2.89**	**0.81**
Sw.Com.t	4	1234	2399	75.85	842	5249	5.48	0.99
	9	1344	1913	80.19	898	5195	5.85	0.99
	14	994	2110	66.28	667	6092	4.22	0.91
	Average	**1191**	**2141**	**74.11**	**802**	**5512**	**5.18**	**0.97**
Sw.Com.c	5	649	1419	36.69	483	6766	4.06	0.95
	10	629	1298	38.09	485	7041	4.05	0.96
	15	1226	2308	72.41	879	6223	5.25	0.98
	Average	**835**	**1675**	**49.07**	**616**	**6677**	**4.45**	**0.96**

The taxonomic assignment of the OTUs performed by Qiime using the Silva (release 128) database, revealed 39 phyla, 110 classes, 245 orders, 483 families and 1,178 genera, obtaining a maximum of 1344 OTUS found, estimated according to Chao1 index as a maximum of 2,399 OTUs, hosting a moderate microbial diversity. Such difference is considering the unique OTUS found known as singletons that are calculated on each sample with Chao's estimation (Colwell *et al.*, 2012). In **Figure 2**, rarefaction curves plot shows that sequencing effort was sufficient to gather a representative number of taxonomic groups for all sample points, since curve slops tends lightly to saturation. Also, these curves show that pile composting samples presented more OTUS as compared to those from the rotoaccelerator process, independently of animal source, swine or avian.

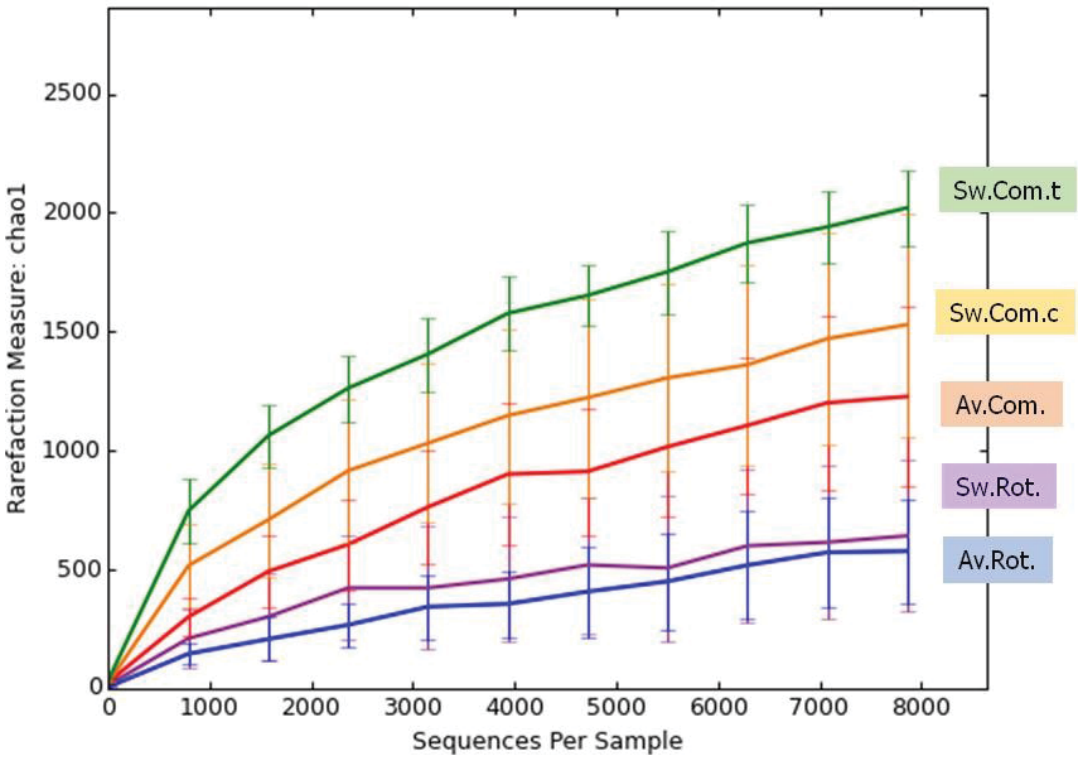


Figure 2. Alpha richness rarefaction curve of 16S gene amplicon sequencing. Number of reads sequences versus the quantity of OTUs calculated on Chaos1 index. Each line represents average of each treatment reaching 7,860 reads. In green: Sw.Com.t (swine composting of adults carcasses), orange: Sw.Com.c (swine composting of young carcasses), red: Av.Com (avian composting), purple: Sw.Rot (swine rotoaccelerator), and blue: Av.Rot (avian rotoaccelerator). Plot generated in Emperor.

The global taxonomic profile, at phylum level (**Figure 3)** reveals in general a similar profile, in which Firmicutes is the dominant group with an average of 68.7%, followed by Actinobacteria with 16.2%, Proteobacteria with 8.5% and Bacteroidetes with 3.4%. Unassigned phylum, that offers potential novel microorganisms, occupied 1.9%, while other phyla represented less than 0.2%. Firmicutes, Actinobacteria and Proteobacteria have been reported as the most abundant phyla during composting processes using different organic wastes by high-throughput sequencing in other studies has revealed (Antunes *et al.*, 2016; de Gannes *et al.*, 2013; Martins *et al.*, 2013; Tashiro *et al.*, 2016; Tkachuk *et al.*, 2014; Tortosa *et al.*, 2017; Zhang *et al.*, 2016).

It is well known that the composting affects bacterial population (Insam and de Bertoldi, 2007) in the same way that practices such as aeration, given by the turning mechanism of substrates, impacts microbial activity and temperature (Antunes *et al.*, 2016). When compared animal source, swine samples presented more abundance of Proteobacteria and Bacteroidetes than avian samples that presented more Actinobacteria and Firmicutes abundance than avian samples. The same comparison applies for long versus short length of treatments. When comparing the type of treatment, traditional composted samples presented higher abundance of other groups than rotoaccelerator samples. Moreover, within the same animal source, traditional composting treatment seems to have more Proteobacteria than rotoaccelerator. So, in general, traditional pile composting indicates higher biodiversity equability at phylum level.

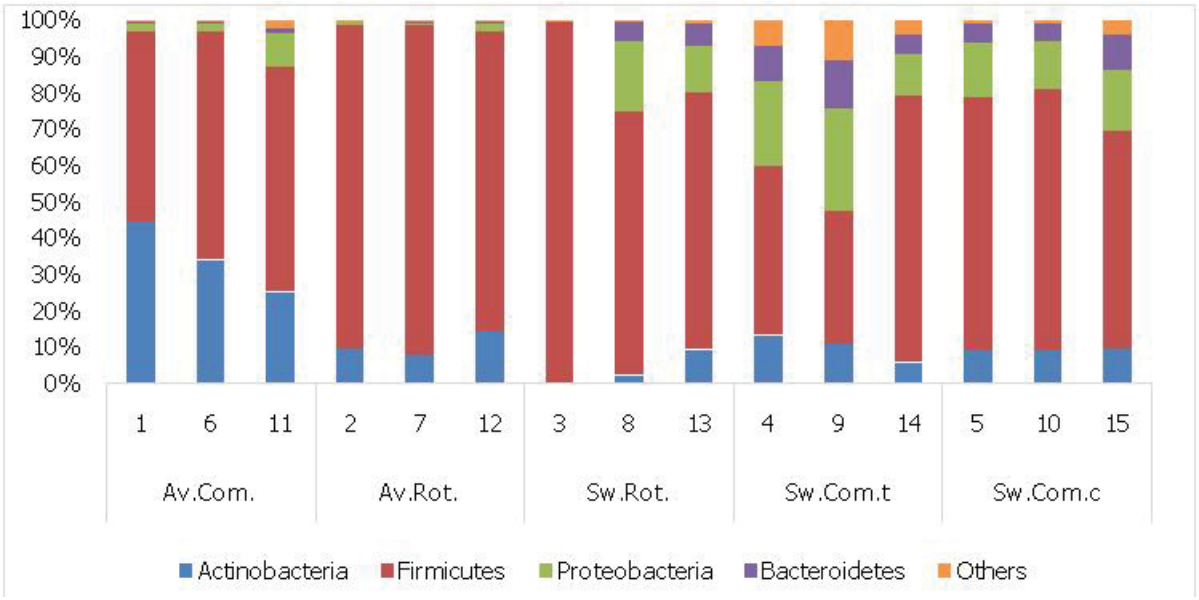


Figure 3. Taxonomic profile of composting metagenome at phylum level. Identification of OTUs was done by sequencing of 16S rRNA gene and comparison against Silva database. In stacked columns are represented the abundances of OTUs in each sample replicate (numbers), distributed throughout the treatments. Colors represent the four major phyla found. Treatments: Av.Com (avian composting), Av.Rot (avian rotoaccelerator), Sw.Rot (swine rotoaccelerator), Sw.Com.t (swine composting of adults carcasses), and Sw.Com.c (swine composting of young carcasses).

At order level, taxonomic profile revealed that the order Bacillales, from phyla Firmicutes, was the most abundant in all treatments, particularly in samples treated by rotoaccelerator (**Figure 4**). On the other hand, the order Corynebacteriales (Actinobacteria) was the most abundant representative of samples containing poultry residues, as well as Flavobacteriales and Sphingobacteriales (Bacteroidetes) from swine wastes. Also, Burkholderiales and Xanthomonadales (Proteobacteria) represented swine samples.

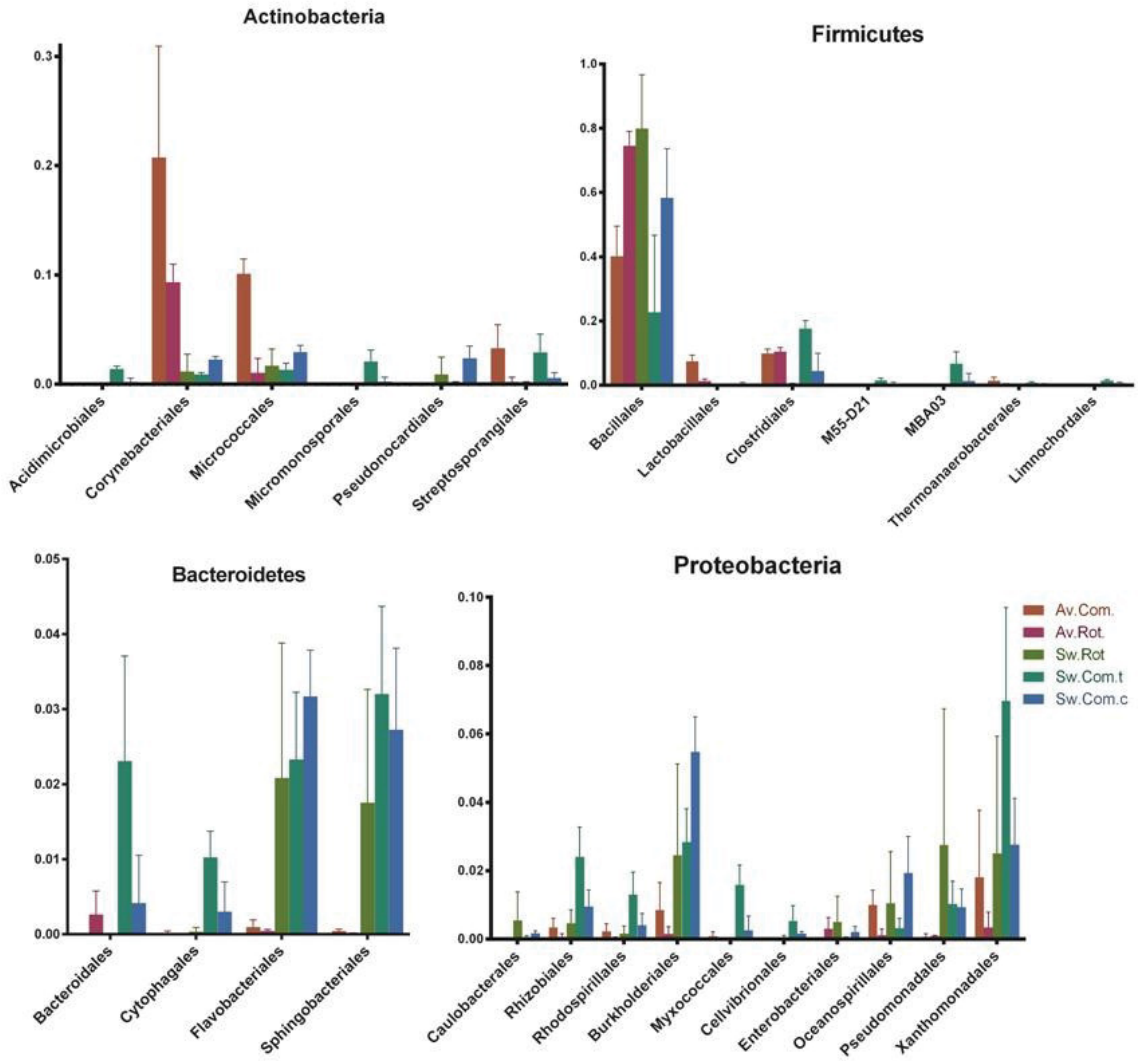


Figure 4. Taxonomic profile of composting metagenome at order level. Identification of OTUs was done by sequencing of 16S rRNA gene and comparison against Silva database. In columns are represented the abundances of OTUs with the major orders found by treatments. Colors represent the treatments: Av.Com (avian composting), Av.Rot (avian rotoaccelerator), Sw.Rot (swine rotoaccelerator), Sw.Com.t (swine composting of adults carcasses), and Sw.Com.c (swine composting of young carcasses).

Besides *Bacillaceae* that counted for the most representative family in all treatments, a pattern of distribution according to treatments was not observed (**Figure 5**). However, uncharacterized OTUS such as unassigned or unculturable counted for an important presence, especially in composting swine samples, unveiling the possibility of finding novel families.

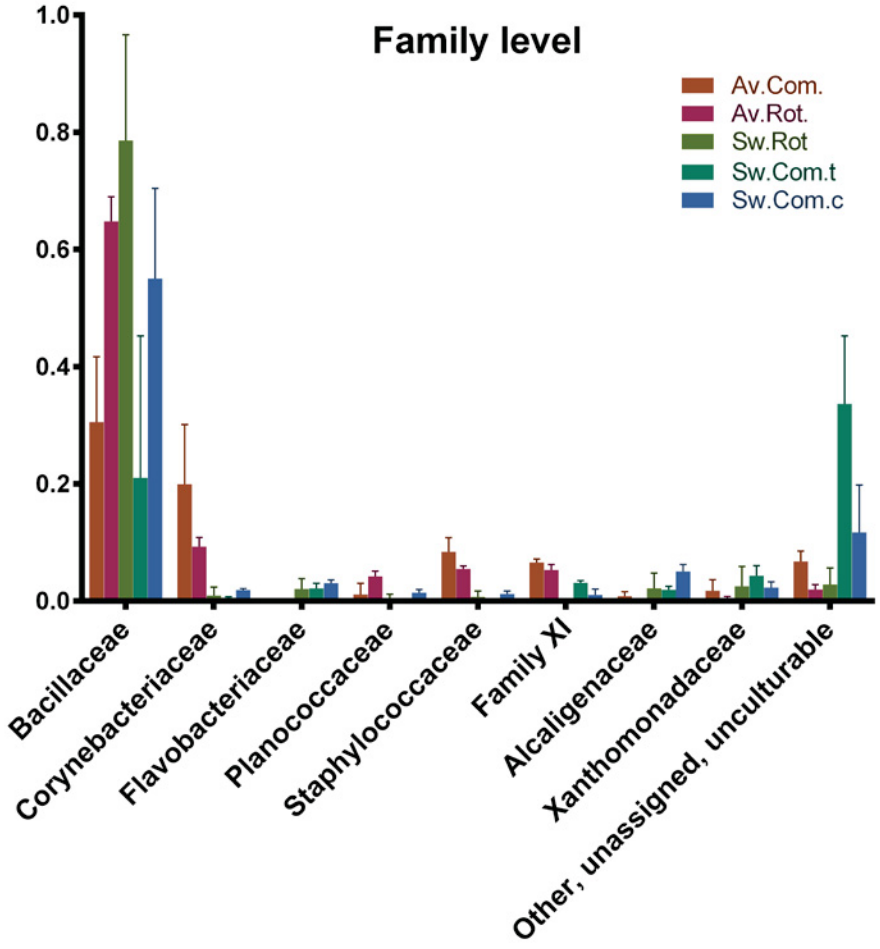


Figure 5. Taxonomic profile of composting metagenome at family level. Identification of OTUs was done by sequencing of 16S rRNA gene and comparison against Silva database. In columns are represented the abundances of OTUs with the major families found by treatments. Colors represent the treatments: Av.Com (avian composting), Av.Rot (avian rotoaccelerator), Sw.Rot (swine rotoaccelerator), Sw.Com.t (swine composting of adults carcasses), and Sw.Com.c (swine composting of young carcasses).

We hypothesized that among the samples there should be differences in terms of microbiome composition regarding the source of animal waste processed and/or the type for composting processing. To assess this hypothesis, multivariate analysis of principal components (PCA) was carried out (**Figure 6**) showing that samples are grouped together according to one of principal components. Principal component 1 (PC1) of 50% comprises an important percentage of the total variances data attributed to animal source indicating that variation in biodiversity samples is primarily representative when

different type of animal source is incorporated in the analysis of composting system (avian or swine). While PC2 in 30% a less important clusterization factor was attributed to the type of treatment (composting or roto-accelerator); and the third component PC3 with only 8% was not attributed to any relevant clusterization component. Then, the plot showed that for composting samples the clusterization best fitted to animal source, being from avian or swine source, than for type of composting, although both samples from roto-accelerator processes are closer related as compared to those from pile composting. This implied that avian samples and swine samples were microbiologically more similar among the replicates, and consequently, traditional composting and rotoaccelerator treatments accounted for more variation.

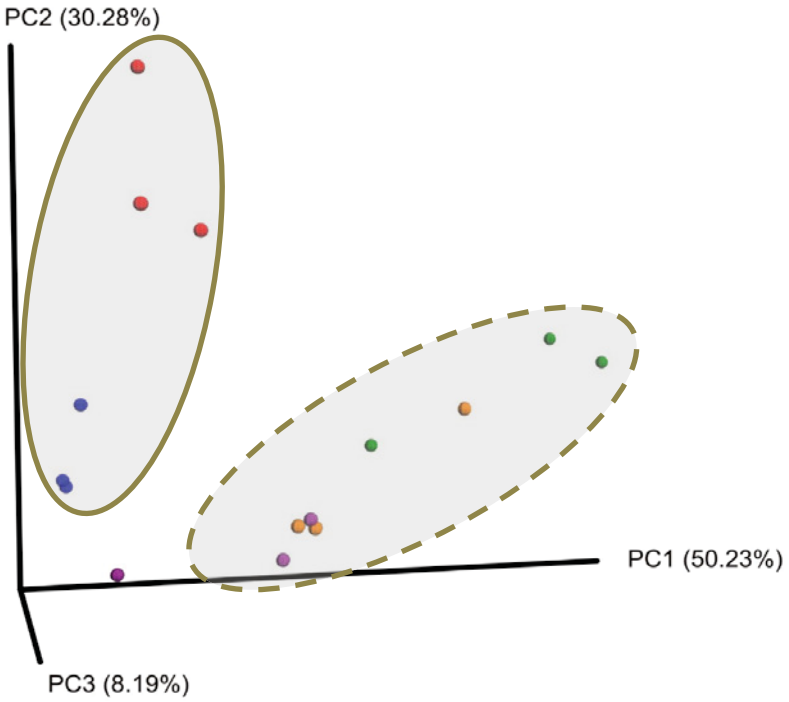


Figure 6. Principal Coordinates Analysis (PCoA) of the bacterial communities, based on weighted Unifrac distance. Each dot represents one sample sequenced. Colors of dots represent triplicates of samples: in **red**: Av.Com (avian composting), **blue**: Av.Rot (avian rotoaccelerator), **green**: Sw.Com.t (swine composting of adults carcasses), **orange**: Sw.Com.c (swine composting of young carcasses), and **purple**: Sw.Rot (swine rotoaccelerator). The circle in continuous line represents the clusterization by source Avian; and in dotted line by source Swine. Modified of plot generated in Emperor.

For a better understanding of biological relevance from animal source in composting processing, characteristic taxonomic groups at level of genera were identified in the two groups of samples, avian and swine sources. This analysis was performed comparing in mean proportion between the two groups along with the associated 95% confidence interval of this effect size and the p-value of the statistical test (**Figure 7**). The genera found associated to avian source were by far *Nasocomiicoccus*, followed by *Tissierella*, *Peptoniphilus*, and *Allofustis*, while in the swine source was *Bacillus*. None of these groups was mentioned in other studies about composting nor treatment of husbandry wastes.

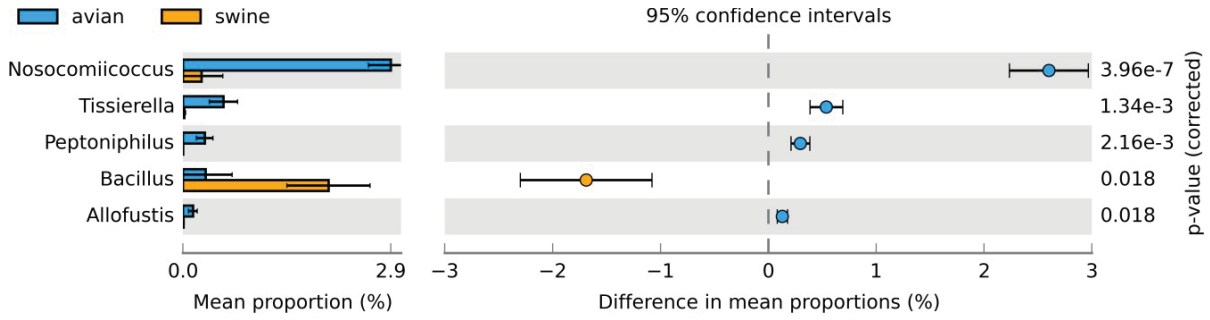


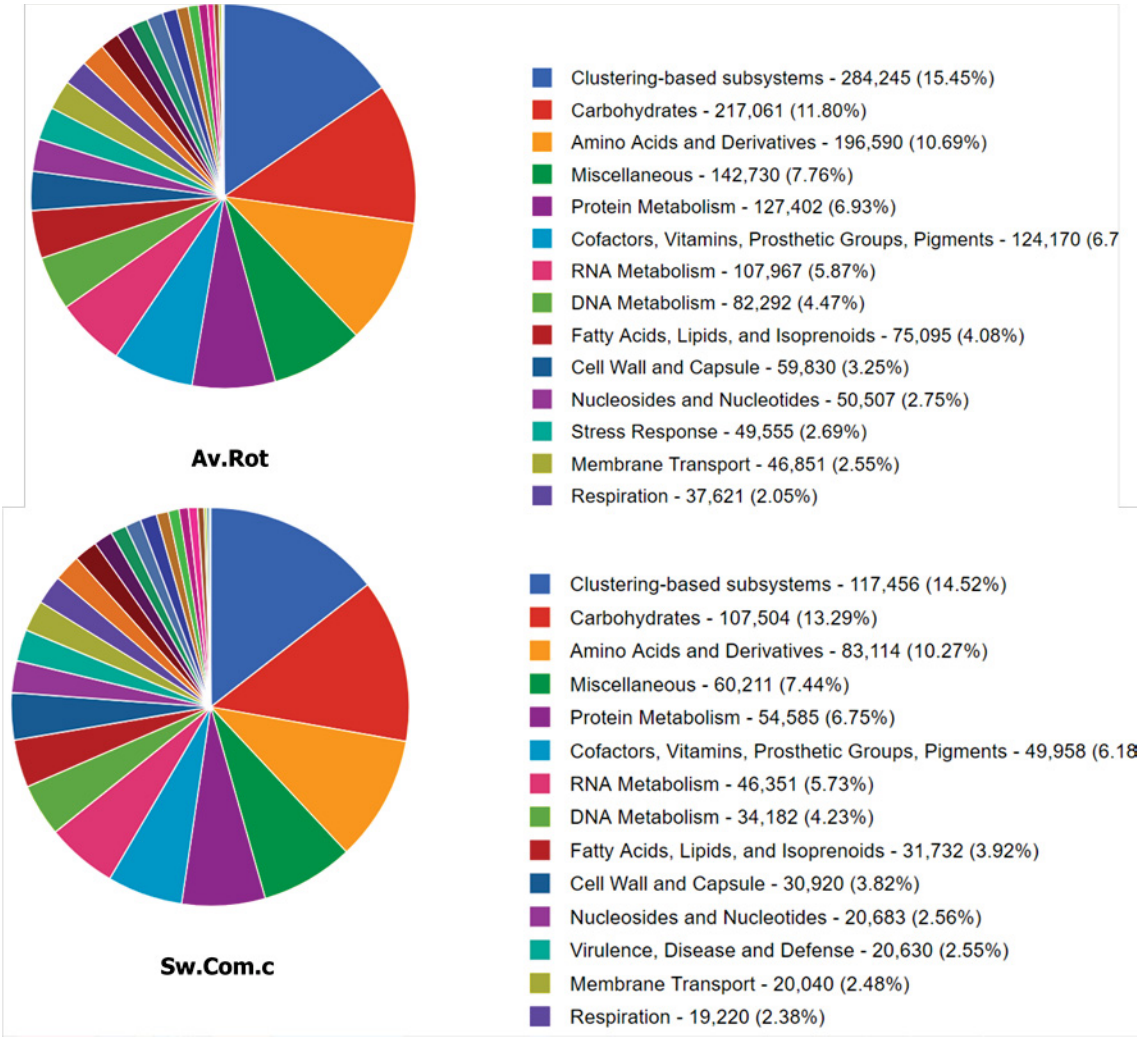
Figure 7. Extended error bar indicating all genera where two sided t-test with confidence interval of 0.95 t-test inverted produces a p-value <0.05. Multiple test correction is done with Bonferroni test. Genera overabundant in the composting systems with avian source represented in blue and with swine sources in yellow.

Two samples were also analyzed by global shotgun DNA sequencing in order to survey the set of genes within these samples (**Table S1**). Global sequencing of total DNA unassembled was submitted to MG-RAST platform. Annotation yielded 85 and 62 % for samples of avian rotoaccelerator (Av. Rot) and young swine composting (Sw.Com.c), respectively (**Table S1**). To distinguish between taxonomic and functional annotation, most of dataset

corresponds to protein sequences or unknown, only 0.60% (5,069 sequences) for Av. Rot and 1.08% (13,687 sequences) for Sw.Com.c codify for rRNA (**Table S1**) useful for OTU classification based on 16S rRNA gene. From this annotation at phylum level both samples confirmed proportions of 16S r RNA gene classification, being Firmicutes highly dominant followed by Actinobacteria, Proteobacteria and Bacteroidetes (**Figure S2**).

The functional profile of composting samples Av.Rot and Sw.Com.c was analyzed in MG-RAST with SEED database subsystem level 1 that provided more number of categories and presented the highest number of annotated sequences among the databases (**Figure S3**). This functional annotation revealed that the two samples presented similar profile with prevalence for clustering-based subsystems and high number of genes involved with carbohydrates, amino acids, proteins, cofactors, vitamins, and others (**Figure 8**), indicating high metabolic capability for composting nutrients. The Av.Rot sample presented genes related to stress response, while Sw.Com.c sample presented in the same proportion genes related to virulence, disease and defense.

Figure 8. Subsystems annotation. Piechart of functional categories annotated for the samples submitted to MG-RAST: Av.Rot (avian rotoaccelerator), Sw.Com.c (swine composting of young carcasses). Analysis performed with SEED-Subsystems database.



4. DISCUSSION

We hypothesized that pile composting process is comparable with that of the roto-accelerator composting in terms of microbiome composition and it could replace the traditional one and take advantage of the higher rate of biotransformation and making more clean and efficient the composting process in field.

Physico-chemical conditions of all samples correspond to given parameters of composting optimization. According to Bernal *et al.* (2009), Carbon-Nitrogen ratio is adequate in the range of 25-35. Higher ratios make the process very slow, and in lower ratios there is an excess of nitrogen, which may be lost by ammonia volatilization or leaching. In our study, sample from swine rotoaccelerator (Sw.Rot.) presented the lowest ratio. However, on the farm the composting material is kept closed in the roto-accelerator, which avoids ammonia volatilization, allowing higher concentration of nitrogen compounds until the end of the process, resulting in a final product with higher value as plant fertilizer (*comm. pers.* Pedro Pies).

Long periods of composing treatment, as seen in the sample for swine rotoaccelerator (Sw.Rot.) should not be necessary when composting is mature and stable (Bernal *et al*., 2009). However, the roto equipment also functions as a stocking chamber for composting, even organic material is processed in 7 days, the compost remains inside being mixed with new material incoming, and several days later, is finally disposed when the equipment is completely full (*comm. pers.* Pedro Pies).

Values of pH near to neutral at the end of the process after having an alkaline peak, is apparently a result of the high occurrence of lactic acid bacteria in combination with ample fermentable sugars which are broken down to form lactic acid and other organic acids, plus carbon dioxide and ethanol in oxygen limited conditions (He *et al*., 2013; Partanen *et al*., 2010). A pH of 7.2-

8.7 as was in our study supports high microbial activity during composting.

Rarefaction plots and biodiversity estimations in our study showed that disturbed environments present higher richness and a greater number of taxonomic groups. That was the case of traditional pile composting that is exposed more directly to environmental conditions such as wind, moisture and temperature seasonal changes that act as stress factors, in contrast to rotoaccelerator samples in a more stable and isolated process in which smooth and controlled movement is part of the process in a closed chamber. This kind of phenomenon is also presented when compare other disturbed environments such as mangrove (Mendes and Tsai, 2018).

At highest taxonomic level, Firmicutes was the vast dominant phylum in all samples. Other composting studies also observed this group in high abundance (Oakley *et al.*, 2013; Tkachuk *et al.*, 2014), and it seems that regardless the composting treatment, substrate composition or temperature, it plays an important role in degrading organic matter throughout de composting process. Actinobacteria, the second most abundant group, importantly form part of the thermophilic solid phase highlighting its potential in cellulosic degradation (Heiss-Blanquet *et al.*, 2016; Martins *et al.*, 2013), found abundantly in the vegetal substrates of litter in composting matter, and being presumably responsible for C/N balance. Proteobacteria is frequently found in mesophilic lignocellulose-degrading microcosms, such as mangrove, soil or sugarcane bagasse, but also at initial phases of composting. Bacteroidetes are known plant cell wall polysaccharides degraders, especially in termite, herbivore and human gut microbiota. Put together, in the hydrolysis of polysaccharides within the compost habitat, there is a synergistic action of Actinobacteria with Proteobacteria and Bacteroidetes (Wang *et al.*, 2016).

In a description of the molecular process sketch during lignocellulose degradation by composting described by Antunes *et al.* (2016), the order Bacillales plays an important role, and Clostridiales secondly important, during initial phases; while the Actinomycetales order plays an important role during

final phases after promoting aeration by turning process. Orders from Bacillales are abundant in thermophilic stages degrading complex polymers such as cellulose and solubilize lignin.

Genus *Noscomiicoccus* from the family Staphylococcaceae was found as the most significant overabundant group in avian source samples of composting and it could be used as marker of poultry waste composting. This genus was first described by Alves *et al.* (2008) on the basis of 16S rRNA gene sequence and phenotypic analyses. Cells are non-pathogenic, aerobic, mesophilic, slightly halophilic and weakly or not- fermentative (Alves *et al.*, 2008). At present, this genus only comprises two species with genomes already sequenced: *N. ampullae* (Alves *et al.*, 2008) and *N. massiliensis* (Mishra *et al.*, 2013), both isolated from clinical samples, from the surface of saline bottles used for washing wounds in hospital wards and from the fecal flora of an AIDS-infected patient. Despite of being poorly reported, genus *Noscomiicoccus* was also listed among 50 genera common to fecal, litter, and carcass samples of commercial poultry (Oakley *et al.*, 2014; Oakley *et al.*, 2013), and also in this study holds for an important place in agricultural samples, specially to characterize avian samples.

The second important genus, significantly overabundant in avian source samples of composting, is the genus *Tissierella.* It belongs to Family XI or family Tissierellaceae as proposed by Alauzet *et al.* (2014), with cells obligately anaerobic, weakly or non-fermentative, creatine or creatinine are required for growth, and typically produce acetate, ammonia and CO_2 (Shah and Hookey, 2015). To date, this genus comprises four species, *T. praeacuta* and *T. carlieri,* reported from various clinical sources; and *T. creatinini*, and *T. creatinophila*, recovered from environmental samples (Alauzet *et al.*, 2014). Currently, only *T. creatinophila* and *T. praeacuta* have their genomes sequenced.

Together in the same family with *Tissierella*, genus *Peptoniphilus* has a close phylogenetic relationship (Alauzet *et al.*, 2014) and significantly

overabundance in avian source samples of composting. Cells are also obligately anaerobic, use peptones and oligopeptide as major energy source (Ezaki and Kawamura, 2015). It comprises several genera, having 15 genomes sequenced and most of them were isolated from clinical specimens.

Genus *Allofustis*, from family *Carnobacteriaceae*, from the order Lactobacillales, has only one specie *A. seminis* that has its genome sequence and it is poorly reported. Cells are facultatively anaerobic and were isolated from porcine semen (Collins *et al.*, 2003). Phylogenetic analysis using 16S rRNA gene sequences showed that this monophyletic group forms a suprageneric cluster that should be resolved when additional species were isolated and described, but it can be distinguished from its close phylogenetic relatives using a combination of morphological, biochemical and chemotaxonomic criteria (Lawson, 2015).

The *Bacillus* genus was found as the only group with significant overabundance in swine source compost samples, and its family Bacillaceae is the most abundant group in this study with 50% average among all samples. This genus with numerous species counts with 180 sequenced genomes. In general, *Bacillus* is a Gram-positive cell, spore-forming, obligate aerobes or facultative anaerobes, and ubiquitous in nature, reported as pathogenic, environmental, and used for industrial purposes (Alcaraz *et al.*, 2010). In composting, this group becomes dominant at the thermophilic stage, and has the ability to produce enzymes involved in polysaccharide and lignocellulosic compounds degradation (Martins *et* al., 2013; Tortosa *et al.*, 2017).

In other less abundant genera found, *Clostridium* and its relatives include anaerobic or micro-aerophilic species, and have been reported to play an important role in landfill cellulose degradation (Zhang *et al.*, 2014). Presence of *Clostridium* and Clostridium-like sequences indicated oxygen limitation (Partanen *et al.*, 2010), as well as cellulose degradation (Zhang *et al.*, 2014), and was found among the 30 most prevalent groups at genera level (data not shown). *Lactobacillus* presence is correlated with low pH (4.7-5.9), mesophilic

temperatures, presence of available carbohydrates, and almost ubiquitous environments was reported in studies with related samples such us composting of a zoo park operation, organic municipal waste composting and animal feces (Martins *et al.*, 2013; Partanen *et al.*, 2010; Endo *et al.*, 2010). The presence of this *taxon* could infer some kind of resistance to a wider pH range and temperature, anyhow consistent with carbohydrate availability at late stage of composting.

Pathogenic and opportunistic enterobacteria genera such as *Salmonella, Campylobacter,* and *Listeria* were not found, suggesting the use of composting treatments for adequate sanitary treatment. Some other genera that may contain pathogenic species include *Escherichia-Shigella*, close related genera that represented 0.54% among all samples. *Pseudomonas* genus was present in swine composting samples (Sw.Rot, Sw.Com.t, Sw.Com.c) with 9.13% average; *Streptococcus* was found with up to 1.22%; and *Staphylococcus* was found not representative with less than 0.79%.

Some authors found significantly correlated an increasing number of genera *Planomicrobium* and *Ohtaekwangia* during maturation phase suggesting as possible biomarkers for this last phase of composting (Tortosa *et al.*, 2017). In this study those groups were found in low prevalence of less than 0.02% or not found. Neither was genus *Thermus*, indicator of thermophilic phases.

Regarding bacterial population over imposing, Partanen *et al.* (2010) indicated that rapid turnings of the composting material produced a fast bacterial cell disintegration including genomic degradation, so this should influence in the content of DNA extracted and amplified, and only further transcriptomic studies could clarify whether population debris of first mesophilic phase are masking populations of the thermophilic phase. According to Tortosa *et al.* (2017), microbial communities in the mesophilic and mature phases are different. In this study, as thermophilic group *Bacillus* counted for the greatest *taxon* found, it could be masking other mesophilic groups.

An increase in diversity was not observed in mature compost, as was observed by de Gannes *et al.* (2013), on the contrary when longer periods of composting expected higher biodiversity, shrinkage on the community diversity was also observed by Zhang *et al.* (2016). However, as authors mentioned, patterns may vary on the feedstock composition, mechanism of composting and the method of analysis.

5. CONCLUSION

Regarding bacterial community structure and physic-chemical parameters, composting process carried on roto-accelerator equipment is comparable with that on static pile composting. Despite traditional pile composting presented slightly higher biodiversity richness, both processes are characterized by microbiota that transforms animal waste together with vegetal substrates in organic matter highly nutrient and pathogen clean, useful for crop fertilization.

In taxonomic profile at level of phylum, Firmicutes dominated all samples followed by Actinobacteria, Proteobacteria and Bacteroidetes. At level of genera, we found characteristic groups according to animal source, *Nosocomiicoccus*, *Tissierella*, *Peptoniphilus* and *Allofustis* for avian wastes, and *Bacillus* for swine waste source composting.

Many genera not previously associated with composts were identified in these systems. Thus, this study contributed with new concepts to compost microbiology and could have implications in monitoring compost process and in the development of biomarkers.

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7. SUPPLEMENTARY INFORMATION

Figure S1. Rényi profile plot of diversity. Visualization of comparison among different diversity indexes. The Y axe shows proportional values of richness and the curve pattern reveals relative abundance of maximum equability when curve tends to horizon. Intersecting curves indicate non-comparable samples. Samples 1, 6 and 11 correspond to Avian Composting; samples 2, 7 and 12 to Avian Rotoaccelerator; samples 3, 8 and 13 to Swine Rotoaccelerator; 4, 9 and 14 to Swine Composting adults; and 5, 10 and 15 to Swine Composting young.

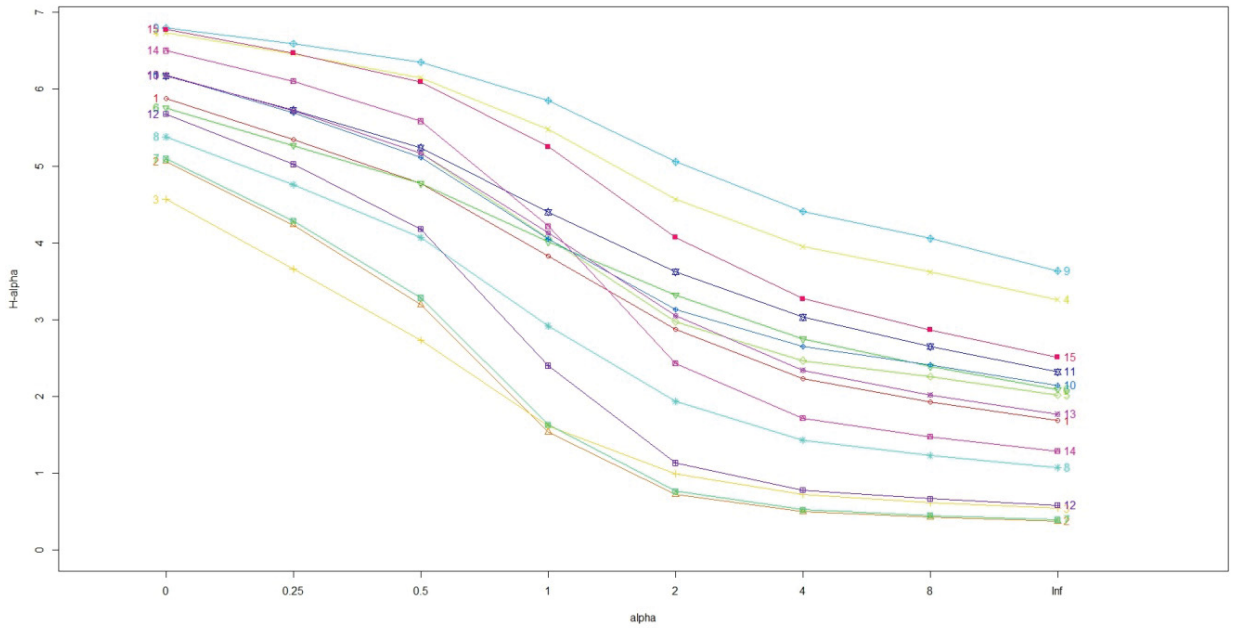


Table S1. Shotgun metagenome summary results. Metagenomes libraries submitted to MG-RAST analysis: Avian Rotoaccelerator (Av.Rot), and Swine composting young individuals (Sw.Com.c).

	Av.Rot	Sw.Com.c
Sequences	1,385,175	956,597
Basepairs	395,835,271	277,383,627
Failed QC	46,443 sequences (3.35%)	54,847 sequences (5.73%)
Mean Sequence Length	221 ± 97 bp	220 ± 101 bp
Ribosomal genes	13,687 sequences (0.6%)	5,069 sequences (1.0%)
Predicted proteins with known functions	1,072,616 sequences (84.88%)	523,583 sequences (61.92%)
Predicted proteins with unknown function	177,423 sequences (14.04%)	316,987 sequences (37.48%)

Figure S2. Taxonomic profile at phylum level. Bars represented percentage of OTUS at different samples treatments: Avian rotoaccelerator (Av.Rot) and Swine Composting of young individuals (Sw.Com.c).

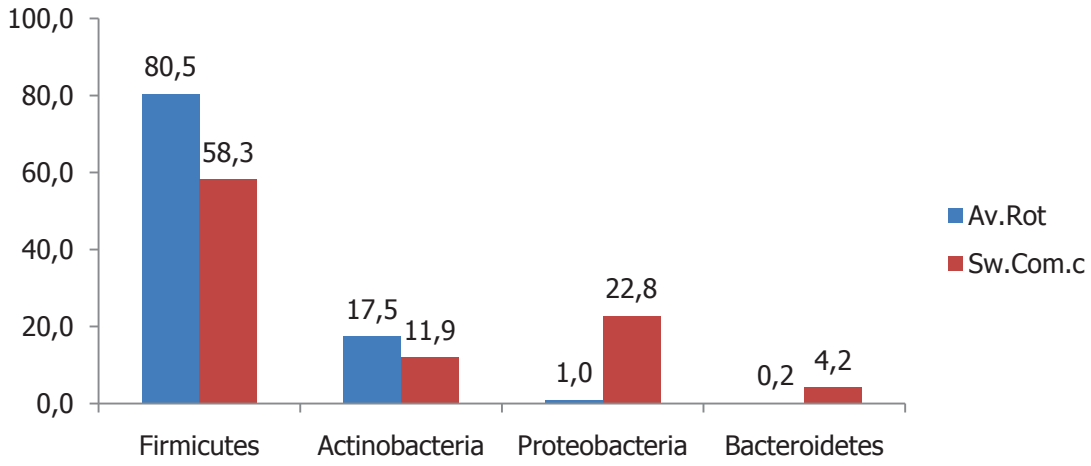
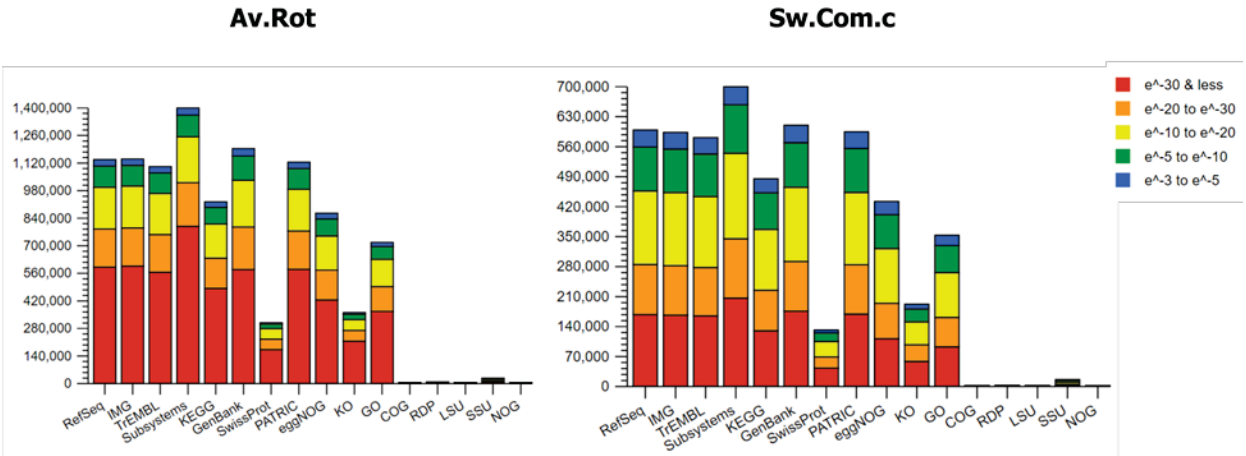


Figure S3. Databases annotation. Columns represent abundance of the estimate number of sequences that contain a given annotation by the different functional databases. Colors represent E-value ranges of the match of a given annotation. Analysis performed in MG-RAST.



CAPÍTULO II

Bioprospecção de lipases na metagenômica: triagem funcional versus triagem *in silico*

Observação: As informações aqui apresentadas correspondem ao trabalho desenvolvido em conjunto com vários pesquisadores que utilizaram as técnicas da metagenômica através das estratégias funcional e *in silico*. O interesse em apresentar as duas estratégias se justifica pela apresentação das vantagens e desvantagens dentro do objetivo comum da bioprospecção metagenômica. O capítulo a seguir apresenta unicamente os resultados obtidos para a estratégia da triagem *in silico*, trabalho correspondente a essa tese de doutorado. Os resultados referentes à lipase identificada através de análise funcional correspondem a dados obtidos pelo doutorando Andre Ferreira Mota. A triagem *in silico* envolveu a participação das Drs. Sarah Sacks e Maura Gueiros nas etapas de sequenciamento e análise das sequências. Minha participação neste trabalho envolveu os ensaios de atividades da enzima Lip720.

Bioprospection of lipases in metagenomics: functional screening versus *in silico* screening

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ABSTRACT

Two lipolytic enzymes were obtained from a metagenomic DNA library constructed from a sample of soil contaminated with animal fat. Enzyme LipE7 (31 kDa) was identified and purified along with its chaperone, ChapE7 (20 kDa), through functional screening using culture media containing 1% tributyrin, trioctanoin or triolein. The other enzyme, Lip720E (47 kDa), which does not require a chaperone, was obtained using an *in silico* screening. A pool of 32 metagenomic fosmids isolated from clones with fat acids hydrolyzing activity on agar medium was sequenced using the Ion Proton platform. DNA sequences were analyzed using MG-Rast for searching of lipase or esterase domains. These proteins were assessed for lipase activities on different fatty acids substrates and stability conditions. Comparison of both screening approaches was addressed.

Key-words: bacterial lipase, metagenome, enzyme screening

1. INTRODUCTION

Lipases are hydrolase enzymes that catalyze hydrolysis of carboxylic ester bonds in the presence of water (E.C.3.1.1.3.). Different from esterases, true lipases act on long chain triacylglycerols, have interfacial activation instead Michaelis-Menten kinetics and prefer hydrophobic substrates (Arpigny and Jaeger, 1999; Bornscheuer, 2002).

Lipases have characteristic alpha/beta folding structure. The catalytic center consists of three catalytic residues: nucleophilic residue (serine, glutamate or aspartate), catalytic acid residue (aspartate or glutamate) and histidine residue. The nucleophilic serine residue usually presented in the conserved pentapeptide G-X-S-X-G (Jaeger et al., 1994; Arpigny and Jaeger, 1999). According to sequence identity and biochemical properties lipases and esterases are classified into eight canonical families (I-VIII), but recent studies reveal more than nine novel families (Lee, 2016; Lenfant et al., 2013).

Bacterial lipases have been gaining importance in biotechnology because of their activity in extreme temperature, pH, osmolarity and presence of detergents and organic solvents. Advantages of microbial lipases include shorter generation time than eukaryote lipases, easier manipulation, scale up and purification, broader substrate specificity, enhanced stability and lower production costs (reviewed in Nagarajan, 2012).

The screening method for assessing cells carrying lipolytic activity by using tributyrin agar plates is still widely used allowing an easy access to lipolytic activity through translucent halos formed around the cell colony. Similar activity detection can be observed using a protein extract. Halo diameter can be used as a semi quantitative parameter for enzyme activity. Other methods include chromophoric substances such as *p*-nitrophenyl esters or naphtyl esters (Popovic, 2017).

Lipases have been isolated using traditional microbial cultivation methods. However, the increasing demand for novel and more diverse enzymes have favored searching for alternative methods. Considering that about 99% of the bacteria species is mainly unknown, a great potential for new enzymes upon environmental and unknown bacteria is presented (Culligan et al., 2014). Metagenomics, which address the environmental genomic DNA without the requirement of growing cells, can be used for biotechnological prospection of novel enzymes, metabolites and products (Schloss & Handelsman, 2003). Metagenomics is considered the most promising methodology for identifying innovative biocatalysts from environmental DNA (eDNA) (Lorenz and Eck, 2005).

Different environments have been explored in prospection for new enzymes using metagenomics approaches. Those environments include compost (Martins et al., 2015; Nurhasanah et al., 2015), soils (Glogauer et al., 2011; Faoro et al., 2010), marine and freshwater environments, vegetal and animal tissues (further revision in Lopez-Lopez et al, 2014; Popovic et al., 2017).

Two different approaches have been described for screening new enzymes using environmental samples: a functional screening in which enzyme activity is detected, and *in silico* screening which is based on DNA sequencing analysis and comparison to databanks.

Functional screening consists on cloning isolated eDNA into vectors allowing protein expression in a determined host, and then these metagenomic DNA library clones are submitted to massive screening for specific activities. This procedure involves that the eDNA can be expressed in the host cell and the activity can be observed and measured (Schloss & Handelsman, 2003).

Strategy based on *in silico* bioprospection is not dependent on DNA cloning since high throughput DNA sequence can be used. Data from DNA sequence are analyzed using homology search through different databank and bioinformatics tools (Schloss & Handelsman, 2003).

Both strategies present advantages and disadvantages, and the choice depends on different circumstances, mainly relying on availability of technical resources at "wet" lab or *in silico* lab.

Lipid degrading enzymes are among the most widely studied with metagenomics (Vorapreeda et al., 2016), because of its multiple biotechnological applications as catalyzers, including stability in organic solvents, wide substrate specificity, stereo-selectivity, and positional selectivity.

In this work the two types of screening for lipolytic activity using eDNA have been addressed. The steps required for each one are detailed and the advantages are discussed. The comparison is based on two successful case studies of lipases LipE7 and Lip720E. Other unsuccessful cases are also presented for discussion.

2. METHODS

2.2 Sequence screening steps for Lip720E

2.2.1 Sequencing of metagenomic library

A metagenomic DNA library constructed using soil from an animal fat effluent pond of a dairy industry (Glogauer *et al.,* 2011) was screened for lipase activity on LB-agar medium containing 1% triolein, allowing identification of potential lipolitic clones. A pool of 32 metagenomic clones had their fosmid DNA isolated and processed for high throughput DNA sequencing. Isolated pooled DNA of those clones was mechanically fragmented using a COVARIS system. Fragments were end repaired, linked to P1 and P2 adaptors and selected by magnetic spheres. This material was sequenced using the Ion Proton platform (Life Technologies) and the CLC Genomic Workbench was used for data analysis and *de novo* assembly. Obtained contigs were screened with MGRast

for lipases and ORFs were identified with FramePlot software. Lipases domains were identified using BLASTp and InterProScan. Amino acid sequence was evaluated for identity against NCBI database, tested for signal peptide on SignalP, and transmembrane helix on TMHH.

Primers were designed to amplify putative lipases present in the original pool of metagenomic DNA. When amplification was unsuccessful, putative lipase genes were synthesized at Integrated DNA Technologies (www.idtdna.com). In order to allow cloning into the pET28 vector, restriction enzymes sites were added. Synthesized lipase gene was digested with *Xba*I and *Hind*III and cloned into pET28a expression vector. Selection of clones was done by kanamycin antibiotic resistance in TOP10 *E. coli* cells.

2.2.2 Expression of recombinant Lip720E and activity measurement

E. coli BL21(DE3) cells carrying the pET28a-lip720E plasmid were grown in 5 L LB medium at 37°C until an OD_{600} of 0.4 and induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.3 mM. The induced culture was incubated for a further 16 h at 16 °C before harvesting by centrifugation (10,000 × g for 5 min) at 4 °C. Cell pellet was resuspended in 120 mL of buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl) and disrupted by ultrasonication in an ice bath (10 cycles of 10 s pulses, with 10 s interval at 40% power) using a SONICATOR® XL 2020 (Heat systems-Ultrasonics Inc.). The crude extract was then centrifuged at 15,000 ×g for 10 min at 4 °C to pellet cell debris. The supernatant containing the protein was maintained at 4°C until use. Protein fractions were analyzed by SDS-PAGE (Laemmli, 1970).

The soluble fraction of the crude extract of Lip720E had its activity tested by visualization of a clear halo of hydrolysis degradation in agar plate supplemented with trybutirin 1 %. Also activity was tested by measuring at 410 nm of the amount of p-nitrophenol released from the p-nitrophenyl ester at 30 °C for 5 minutes. The substrate solution was composed of 20 mM of p-

nitrophenyl decanoate (pNPD) in acetonitrile/isopropanol (1/4 v/v) with a buffer 10x containing Tris-HCl pH 8 500 mM, $CaCl_2$ 10 mM and Triton X-100 3%, under agitation in a water bath at 60 °C, until the solution became transparent. Then 12.5 µL of the substrate solution was pipetted into a 96-well microtiter plate and the reaction was initiated by addition of 20 µL of the crude cell extract containing the enzyme. With the final volume of 250 µL, the reaction mixture contained Tris-HCl 50 mM pH 8, $CaCl_2$ 1 mM, Triton X-100 0.3 % (v/v), pNPD 1 mM, isopropanol 4 % (v/v), acetonitrile 1 % (v/v) and crude extract. All experiments were performed in triplicate, the extinction coefficients of p-nitrophenol were determined under each reaction condition and the effect of non enzymatic hydrolysis of substrates was subtracted. One unit of lipase activity was defined as 1 µmol of p-nitrophenol produced per minute.

Determination of enzyme specificity was done using seven different substrates: p-nitrophenyl acetate (C-2); p-nitrophenyl butyrate (C-4); p-nitrophenyl valerate (C-5); p-nitrophenyl caproate (C-6); p-nitrophenyl decanoate (C-10); p-nitrophenyl dodecanoate (C-12) and p-nitrophenyl myristate (C-14). Lipolytic activity was determined using 3 µL of supernatant extract with 1 mM pNP-esters in acetonitrile:isopropanol (4:1) during 5 minutes.

2. RESULTS

2.2 Lip720E: a lipase obtained from sequencing analysis of a metagenomic library

Glogauer *et al.* (2011) used a sample from an industry waste lagoon containing animal fat. This sample was used to construct a metagenomic DNA library using a fosmid as vector. This metagenomic DNA library had about 500 thousand clones and it has been used for screening new lipases enzymes (Glogauer *et al.*, 2001; Martini *et al.*, 2012, 2014).

In order to evaluate an *in silico* screening approach to identify a new lipase activity, 32 metagenomic clones, previously selected for lipase activity on agar containing 1% triolein (Gloglauer *et al*., 2011) were pooled and submitted to a high through put next generation sequencing using the Ion Proton platform (Life Technologies) as described in methods.

Sequences of about 100 bp were processed yielding libraries SLP1 with 68,483 reads and SLP2 with 153,413 reads. *De novo* assembly resulted in 23 and 30 contigs with N50=3,717 and 5,550, respectively for SLP1 and SLP2, with more than 1,042 bp. Those sequences were analyzed for the identification of those potentially codifying for lipase/esterase hydrolases. From these sequences some were identified as incomplete sequence showing only hydrolase domains.

The contig 720 was found to encode two genes potentially related to lipase/esterase functions (here namely lip720E and lip720G). The lip720E gene showed 99% identity with a non-characterized esterase from *Acidovorax caeni* (Heylen, Lebbe and De Vos, 2008), and 78% identity with an esterase/lipase from unculturable bacteria named TB_I_H8_p (GenBank KM669734.1). Amino acids sequence did not show a signal peptide nor a transmembrane helix. The lip720G gene coded for an enzyme with 97% identity with a lipase from SGNH hydrolases family from *Acidovorax caeni*, and 61% identity with a lipase from *Verminephrobacter aporrectodeae*. Amino acid sequence contained signal peptide and no transmembrane helix.

Attempts to amplify those sequences by PCR were unsuccessful, therefore the gene was chemically synthesized with codon optimization for expression in *E. coli*. Synthesized sequences were digested with restriction enzymes *Xba*I and *Hind*III, and inserted in pET- 28a vector previously digested with same enzymes.

For protein expression, plasmids were transformed in *E. coli* BL21(DE3), however although several attempts were carried out to express Lip720G, the protein seems to be toxic for the bacteria cells since cell death was observed

upon induction. Therefore, characterization was carried out for Lip720E.

Phylogenetic analysis showed that Lip720E belongs to family III of lipases (Jaeger and Eggart, 1999; Lee, 2016). In a phylogenetic tree performed with Clustal Omega (EMBL-EBI), this amino acid sequence was clustered together with the other 5 lipases of the highest identity at Blastp, and they have a close relationship with lipases from canonical family III (**Figure 8**). Sequence analysis shows that Lip720E presents the characteristics of family III that includes typical fold alpha/beta from hydrolases; the catalytic triad for serine hydrolases, identified as Ser_{215} Asp_{372} His_{380} of 432; active-site aspartic residue, identified as Asp_{372}; and conserved pentapeptide of serine catalysis GXSXG on Ser_{215}.

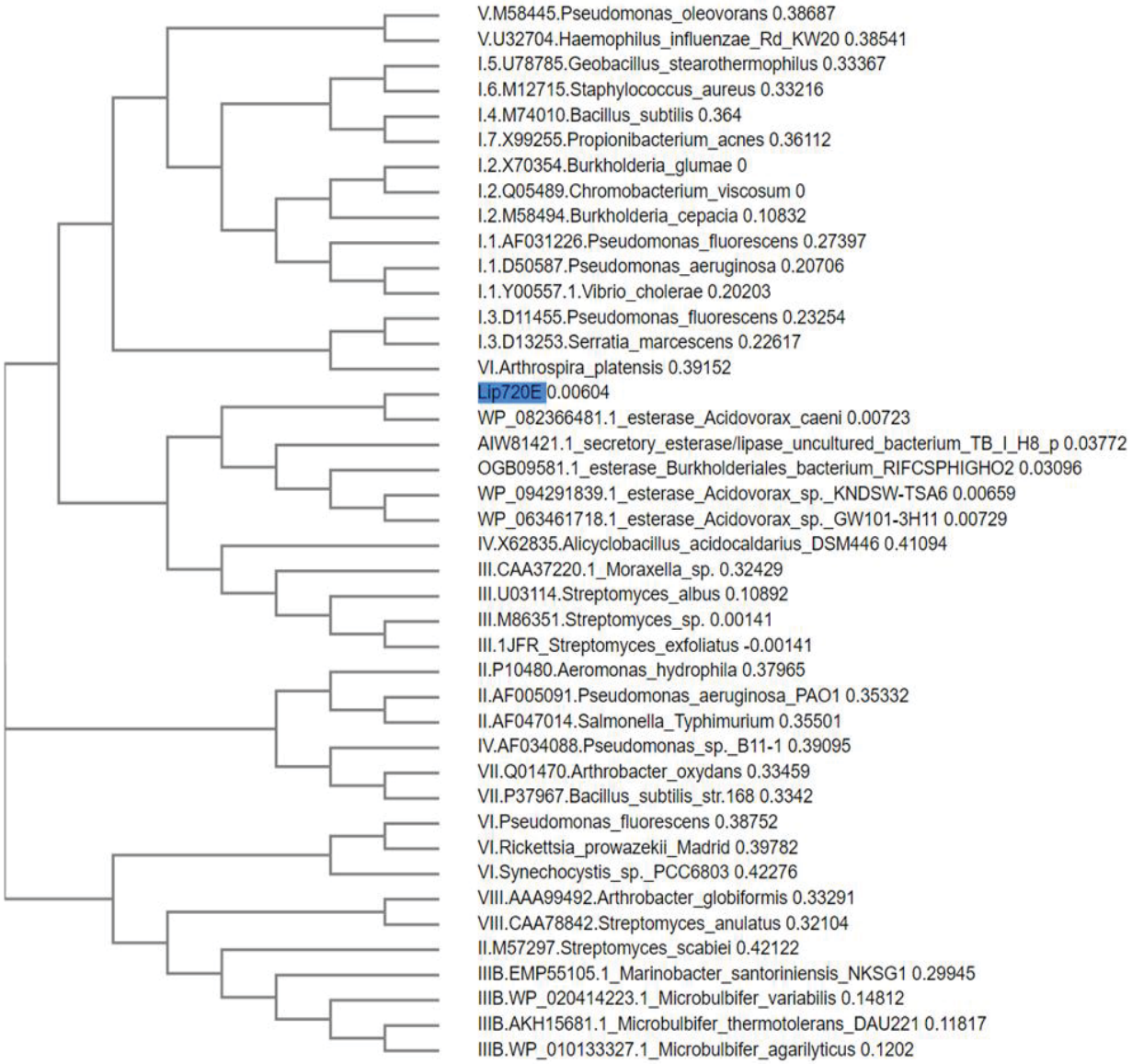

FIGURE 8. Phylogenetic tree with families of lipases. Analysis performed in Clustal Omega (EMBL-EBI) with Neighbour-joining method. Includes Lip720E highlighted in blue and representative members of each family classified by Jaeger and Eggart (1999) indicated in roman letters, also included lipases from a new family related to family III, a sister group IIIB. It is indicated access number of sequence, encoding product for group clustered with Lip720, organism name in genus and specie when available, and distance number of each branch of the tree.

Protein expression of Lip720E was performed at OD_{600} ~0.4 with 0.3 mM IPTG at 16°C overnight, and a low expression was detected. Nevertheless, compared to control, activity was detected on crude extract and supernatant extract using agar plates containing 1 % tributyrin (**Figure 9**) and 1% triolein.

Different attempts for purification of Lip720E were performed however, we were unable to produce a purified protein. Therefore, activity was tested using a cell extract upon induction.

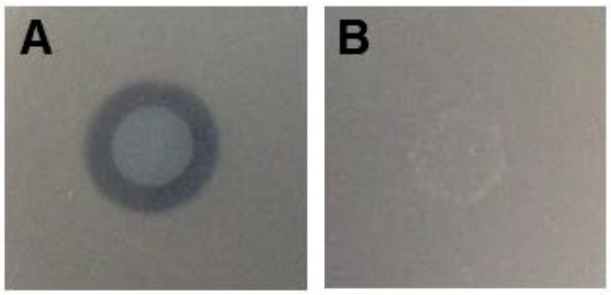

FIGURE 9. Activity of Lip720E on agar plate supplemented with tributyrin 1%. Hydrolysis halo of 15 µL of supernatant extract of induced *E.coli* BL21(DE3), after cell lysis and centrifugation **A.** protein extract of cells carrying plasmid pET-28a-lip720E, diameter: 14.5 mm. **B.** Protein extract of cells carrying plasmid pET-28a without insert. Lipolytic activity was determined using 3 µL of supernatant extract with pNP-esters during 5 minutes, in 1 mM substrate in acetonitrile:isopropanol (4:1).

Hydrolysis activity using pNP-esters with different size chains were carried out (**Figure 10**) showing that Lip720E has lower activity for short chain acids. A higher activity was observed with C10 chain substrate.

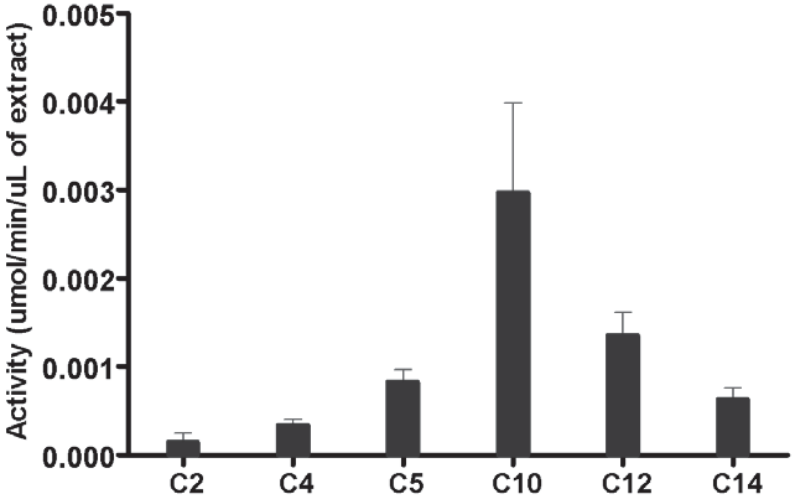

FIGURE10. Lip720E activity in different esters of p-nitrophenyl. P-nitrophenyl acetate (C2), p-nitrophenyl butyrate (C4), p-nitrophenyl valerate (C5), p-nitrophenyl decanoate (C10), p-nitrophenyl dodecanoate (C12), p-nitrophenyl myristate (C14).

3. DISCUSSION

In order to compare two strategies for identification of new lipase, a metagenomic DNA library was screening using a functional and an *in silico* approaches. The functional screening involved the identification of a fosmid clone with activity to produce a hydrolysis halo in tributyrin and triolein containing medium. After selection, fragments of the fosmid DNA were subcloned and subject to a second screening for lipase activity. Subclones showing activity were selected and analyzed. This strategy leaded to the identification of LipE7, a protein with homology with an uncharacterized lipase of *Aeromonas hydrophila*. Moreover, LipE7 is a lipase which requires a specific foldase for correct folding and whose gene frequently found near to the lipase gene. This foldase coding gene was identified in the same subclone of lipE7 allowing us to obtain an activity lipase. Part of these results are described in the dissertation by Andre Ferreira Mota (2016).

In this chapter we described the Lip720E, a lipase identified by *in silico* screening. Lip720E protein was characterized as a lipase for acting on long chain ester substrates and it was found to be active in the water-soluble fraction of cell extract at room temperature. Further studies such as substrate specificity, enzyme stability, product quantification and possible biotechnological application could not be performed since purification of the enzyme from the rest of cell components was not possible, nor its visualization in polyacrylamide gel electrophoreses, steps required for assessing activity.

Prospection based on *in silico* strategy has several vantages considering nowadays capability for high throughput DNA sequencing and computation analyses availability, including different databank and comparison resources. On the other hand, this approach relies on sequence similarity (whole protein or

protein domains) therefore, making more difficult in finding new enzymes with low similarity to those described as well as active enzymes with expected functions.

The problems we faced in this work were related to expression of heterologous protein in a surrogate host since we found our gene to being originally expressed under a codon bias completely different to the selected host *E. coli*. In that case, synthesis of the gene with known codon bias overcame this issue with verifiable expression and activity. However, the syntheses probably resulted in low expression that impaired with low or none visualization in polyacrylamide gel making it difficult the visual verification of expression.

Another obstacle we have faced, not related to the metagenomic strategy screening, was the inability of achieving the His-tag fusion protein purification. Despite the simplicity and efficiency of this affinity purification method we were unable to obtain a purified protein. We have also tried unsuccessfully hydrophobic interaction and ion exchange chromatography.

We also found difficulties in measuring lipolitic catalysis in rapid, precise and comparable manner, with no requirement of purification. Heterogeneous character of lipase catalysis makes difficult to accurately quantify lipolitic reaction, due to its nature of being hydrophilic enzymes and acting over hydrophobic substrates (Beisson *et al.*, 2000). In this study it was used the agar plate containing fatty acid substrate for detecting hydrolytic activity of lipases by halo formation. This method allowed precise identification among few samples, but required few days or at least several hours in order to appreciate visible clear halo. Also it was tested the use of chromogenic substrates, p-NP-esters of different fatty acids, but screening conducts to misleading results since they are not natural substrates (Beisson *et al.*, 2000).

The *in silico* strategy for prospection of the same metagenomic dataset allowed the identification and partial characterization of another lipase that encountered another issue of this strategy. The sequence *lip355* codifies for a

carboxylic ester hydrolase (E.C. 3.1.1) with identity of 77% for a triacylglycerol lipase from *Rhodoferax ferrireducens* T118 (GeneBank WP_011465630.1). Lip335 presents a conserved pentapeptide motif G-X-S-X-G and a catalytic triad, thus, belonging to a true lipase family. The sequence *lip355* was amplified by PCR, cloned in a vector for superexpression and the protein was expressed in *E.coli*. The expressed Lip355 was shown to be insoluble despite different protocols for expression and cell lysis conditions. Further *in silico* and *in vitro* analyses indicated that a specie-specific foldase protein is required for the activity of the lipase Lip335 (Unpublished data).

Although *in silico* prospection may represent an interesting approach for identifying new enzymes, several difficulties are faced. Different codon bias between the original host and the one used for protein expression, requirement of a co-factor or a specific chaperone, for example, can impair activity. Since sequence homology comparison is used in order to identify potential genes, a potential restriction with activity related to a novel sequence may apply. On the other hand, functional screening allows the identification of active enzymes upon expression in a different host, indicating that any requirement for activity (if any) can be provided by the host cell. Even with the requirement of cloning prior the prospection, if activity analyses are necessary, a functional screening may be a more appropriate approach.

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