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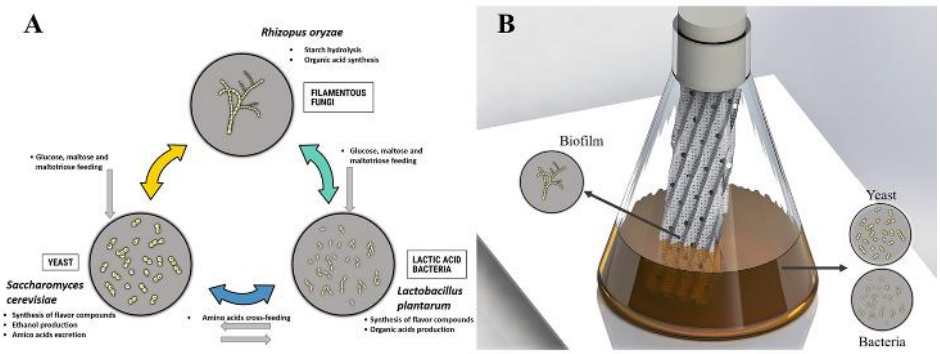
Impact de la composition microbienne sur le profil volatolomique des boissons fermentées cambodgiennes : des communautés naturelles aux communautés synthétiques



Impact of microbial composition on volatolomic profile of Cambodian fermented beverage: from natural to synthetic communities

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Dissertation originale présentée en vue de l'obtention du grade de docteur en sciences agronomiques et ingénierie biologique

Co-promoteur : **Prof. Marie-Laure FAUCONNIER**
Co-promoteur : **Dr. Hasika MITH**
Promoteur : **Prof. Frank DELVIGNE**
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Résumé

LY Sokny (2019). **« Impact de la composition microbienne sur le profil volatolomique des boissons fermentées cambodgiennes : des communautés naturelles aux communautés synthétiques ». (Thèse de doctorat en anglais).** Gembloux, Belgique, Gembloux Agro-Bio Tech, Université de Liège. 177 pages, 10 tableaux, 21 figures.

Résumé

Cette thèse s'intéresse aux problèmes liés au développement de la production de vin de riz au Cambodge. Le choix du ferment starter est l'un des facteurs influents sur le rendement et la qualité des boissons alcoolisées. La composition microbienne des starters varie selon les régions de production et est influencée par l'environnement et les matériaux utilisés. A travers cette étude un premier pyro-séquençage par ARNr pour étudier le microbiote des starters traditionnels cambodgiens a été élaboré. Les résultats du **chapitre 3.1** ont mis en évidence l'impact de la composition du microbiote sur le profil des composés aromatiques synthétisés. Les souches dominantes, principalement, le champignon filamenteux *Rhizopus oryzae*, la levure *Saccharomyces cerevisiae* et la bactérie lactique *Lactobacillus plantarum* ont été identifiés. Il a été rapporté que ces trois souches affectent considérablement le profil en composés aromatiques du vin. Les résultats du **chapitre 3.2** ont détaillé le rôle important de ces trois souches lors du processus de remplacement de la communauté naturelle. Le profil aromatique était complémentaire à celui de la communauté naturelle (*Dombea*); de plus, certains arômes désagréables ont été réduits lors de l'utilisation de la communauté synthétique contrôlée et connue. Une autre partie de l'étude a mis en évidence l'importance du choix du type de dispositif utilisé pour la culture du biofilm afin de favoriser son attachement. Le **chapitre 3.3** a concentré l'évaluation de l'effet du système de fermentation sur l'attachement de *Trichoderma* spp. et la production d'hydrophobins. A cet égard, deux systèmes de fermentation (SFE, DFR) ont été évalués. L'ensemble des résultats a montré l'importance du choix du system de culture biofilms, notamment dans le cas de l'étude des interactions microbiennes. Ainsi le recours à l'utilisation d'un bioréacteur alternatif a permis de comprendre et de mieux contrôler l'interaction ainsi que la structuration de la communauté synthétique étudiée. Les system de culture biofilm ont permis de mieux contrôler la morphologie complexe du mycélium. Les résultats de cette étude pourraient être considérés comme une voie prometteuse pour le développement de la technologie du vin de riz au Cambodge.

Mots clés : fermentation du vin de riz, communautés microbiennes, SPME-GCMS, séquençage de l'amplicon, hydrophobin, physiologie de l'état solide, colonisation de la surface, réacteur à biofilm, interactions microbiennes, volatolomique, fermentation alcoolique.

Abstract

LY Sokny (2019). **« Impact of microbial composition on volatolomic profile of Cambodian fermented beverage: from natural to synthetic communities ». (PhD Dissertation in English).** Gembloux, Belgium, Gembloux Agro-Bio Tech, University of Liège. 177 pages, 10 tableaux, 21 figures.

Abstract

This thesis addressed issues related to rice wine production development in Cambodia. The choice of alcoholic ferment starter is one of the most influencing factors on the yield and quality of the alcoholic beverage. The microbial composition of starters varied according to the regions where they were produced and was influenced by the environment and the material used. Through this study, a first rRNA pyro-sequencing to study the microbiota of traditional Cambodian starters was developed. The results of Chapter 3.1 highlighted the impact of the composition of the microbiota on the profile of the synthesized aromatic compounds. The dominant strains, mainly the filamentous fungus *Rhizopus oryzae*, the yeast *Saccharomyces cerevisiae* and the lactic acid bacteria *Lactobacillus plantarum* have been identified. These three strains have been reported to significantly affect the aromatics profile of the wine. The results of Chapter 3.2 detailed the important role of these three strains in the process of replacing the natural community. The aromatic profile was complementary to that of the natural community (Dombea); moreover, some unpleasant aromas have been reduced when using the controlled and known synthetic community. Another part of the study highlighted the importance of choosing the type of device used for biofilm cultivation to favour its attachment. Chapter 3.3 focused on the evaluation of the fermentation system on the attachment of *Trichoderma* spp. and the production of hydrophobins. In this respect, two fermentation systems (SFE, DFR) were evaluated. All the results showed the importance of the choice of the device for the biofilm culture, especially in the case of the study of microbial interactions. Thus, the alternative bioreactor design makes it possible to understand and better control the interaction as well as the structuration of the synthetic community studied. Biofilm reactors have made it possible to better control the complex morphology of the mycelium. The results of this study could be considered as a promising avenue for the development of rice wine fermentation technology in Cambodia.

Key words: Rice wine fermentation, microbial communities, SPME-GCMS, amplicon sequencing, hydrophobin, solid-state physiology, surface colonization, biofilm reactor, microbial interactions, volatolomic, alcoholic fermentation.

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Table of contents

List of figures

List of figures in chapter 3.1

List of figures in chapter 3.2

List of figures in chapter 3.3

List of tables

List of tables in chapter 3.1

List of tables in chapter 3.2

List of tables in chapter 3.3

List of abbreviations

AF	**:**	Alcoholic fermentation
B.C	**:**	Before Christ
CLSM	**:**	Confocal laser scanning microscopy
DFR	**:**	Drip flow reactor
DGGE	**:**	Denaturing gradient gel electrophoresis
DHS	**:**	Dynamic head-space
Dm	**:**	Dried mass
DNA	**:**	Deoxyribonucleic acid
DPPH	**:**	1,1-diphenyl-2-picrylhydrazyl
DVB/CAR/PDMS	**:**	Divinylbenzene/Carboxen/Polydimethylsiloxane
EPS	**:**	Extracellular polymeric substances
ESI	**:**	Electrospray ionization
FISH	**:**	Fluorescence in situ hybridization
GCMS	**:**	Gas Chromatography Mass spectrophotometry
GFP	**:**	Green fluorescent protein
HPLC	**:**	High Performance Liquid Chromatography
LAB	**:**	Lactic acid bacteria
LPS	**:**	Lipopolysaccharide
MALDI	:	Matrix-assisted laser desorption ionization
MLF	**:**	Malolactic fermentation

OTU	**:**	Operational Taxonomic Units
mRNA	**:**	Messenger Ribonucleic Acid
NS	**:**	Non-*Saccharomyces*
PVC	**:**	Polyvinyl Chloride
RAPD	**:**	Random amplified polymorphic DNA
RNA	**:**	Ribonucleic Acid
SF	**:**	Simultaneous saccharification and fermentation
SPME	**:**	Solid Phase MicroExtraction
HS-SPME	**:**	Head Space Solid Phase MicroExtraction
SSCP	**:**	Single-strand conformation polymorphism
TOF	**:**	Time of flight
YEPD	**:**	Yeast extract peptone dextrose

1

GENERAL INTRODUCTION

Fermented foods and beverages have been famous since ancient times all over the world. All type of alcoholic beverages including beers, wine and spirits are principally subjected for scientific research and this research has been increasingly considered and developed. Starchy-based wine is also an interesting research; especially, in Asian country including China, Thailand, Vietnam, Philippine, India, as well as in Cambodia, yet lack of research in Cambodia.

The text below will describe the important of rice and rice wine in Asia, how they are made, and what are the obstacle of rice wine development.

1.1. Red rice and its utilization for fermented beverages/ food

Approximately in 39 countries, rice is the staple diet, but the dependence on rice for food energy is much higher in Asia than in other regions. Rice provides 35 to 59 percent of energy consumed for 2700 million people in Asia. A mean of 8 percent of food energy is supplied by rice for 1000 million people in Africa and Latin America (FAO, 1984). The genus *Oryza* consists of two cultivated species, *O. sativa* (the Asian cultivated rice) and *O. glaberrima* (the African cultivated rice), and more than 20 wild species, many of which are consumed in the human diet in certain regions of the world (Sompong *et al*., 2011). Pigmented rice is the seed of *Oryza sativa Linne* (Gramineae), and is a type of ancient rice originated in Hanzhong, Shanxi in China and is also called red rice and purple-black rice or black rice. Unpolished pigmented rice has a hard surface and a white inner area, and contains purple black pigments (anthocyanin) in the unpolished seed coat (Sompong *et al*., 2011). Black sticky rice is used as ingredient in many dishes since it has a number of nutritional advantages over common rice. Moreover, the black sticky rice can be used as the raw material to produce wine, dessert, edible vinegar, alcohol, fine noodles, noodles, and fermented black sticky rice. For instance, the fermented black sticky rice is used to mix with many types of fermented fish in order to enhance flavour and taste. Furthermore, black

sticky rice extract is used in confectionery (candies, gum, cookies, pudding, jelly, yogurt, chocolate, and so on), base cosmetics (lotion, milk, cream, and so on), body cosmetics (body lotion, body cream, and so on), cleansing cosmetics (soap, and so on), makeup cosmetics, functional foods, nutraceutical foods, health foods, and anti-diabetic supplement.

In some parts of Bhutan, China, India, Sri Lanka, Philippines, and other Asian countries, red rice is not considered to be a weed but a traditional staple crop. Red rice is also gaining popularity in Japan as a functional food owing to its high polyphenols content. The growing interest for red rice in Japan has resulted in the emergence of various secondary products such as coloured noodles, cakes, and alcoholic beverages. Although widely consumed as white rice, there are many special cultivars of rice that contain colour pigments, such as black rice, red rice and brown rice have been cultivated for a long time in Asia (Sompong et al., 2011; Sutharut et al., 2012). Cambodian people usually call red rice as red sticky rice (*Ankor Damnerb Kmao*), considered to be a noxious weed in the southern United States, Greece, Latin America, Spain, and other temperate regions where irrigated rice is grown. It names is derived from the colour of the dehulled grain which may vary from deep red to pink. Red rice plants are difficult to differentiate from rice cultivars at the seedling stage. However, at tillering, flowering, or maturity stages, red rice plants are recognizable by the following characteristics: hispid, light-green leaves, profuse tillering, longer and more slender panicles and heavy shattering of grains (Diarra *et al.*, 1985). According to Sutharut et al., (2012), red rice is generally considered to be of the species *Oryza sativa* which can be classified into four biotype groups, namely, *O. sativa* spp. *indica*, *O. sativa* spp. *japonica*, *O. nivara*, and *O. rufipogon*.

Starch is considered as main component in rice as well as in red rice. With the sharp increase in lifestyle-related health issues and diseases such as diabetes, cancer, and heart problems; scientists are looking at quality traits other than carbohydrates,

protein, and fat in foodstuffs. Evaluation of foodstuffs now places greater focus on their antioxidant properties, glycemic index, and mineral content (Ahuja *et al.*, 2007).

Scientists are looking at rice as more than just starchy food and are analysing the antioxidant properties of pigmented rice. Also, the mineral content of rice varieties is gaining importance. The greater antioxidant property in red rice was found than black and white rice. With this rediscovery of their nutritive and medicinal value, red rice has begun to regain their old position and prestige. It is now known that flavonoid and anthocyanin compounds are closely related to the anti-oxidation properties of black rice. Moreover, DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity in red rice is higher than in black and white rice, and that this activity is correlated with polyphenols and pro-anthocyanidin content. Considering that the highest scavenging activity due to the presence of polyphenols is in red rice than in black and white rice, red rice could once again find favour with health-conscious consumers. It is high time that people in India took a fresh look at similar properties in the vast pool of indigenous red rice. There is also a dire need for clinical validation of the medicinal value of red rice reported in ancient literature, and for research on food preparations such as poha (flake rice), noodles, and sewai (vermicelli) from red rice. The proximate composition of red rice varieties from different region were summary in **Table 1-1.**

Table 1-1 : proximate composition of red rice varieties (g/100 g dm) (sompong et al., 2011)

Rice sample	Origin	Moisture	Ash	Fat	Protein	Total dietary fibre	Total carbohydrate
Bahng Gawk	Thailand	11.55	1.33	2.86	9.21	3.63	
Haek Yah	Thailand	12.38	1.40	2.91	7.40	4.18	75.92
Niaw Look Pueng	Thailand	11.45	1.50	2.37	7.16	3.17	77.53
Sung Yod Phatthalung	Thailand	9.28	1.42	2.67	10.36	4.51	76.27
Niaw Dawk Yong	Thailand	12.01	1.45	3.19	9.62	3.75	73.73
Niaw Lan Tan	Thailand	13.12	1.26	3.08	7.35	3.27	75.20
Sri Lanka Red Rice 1	Sri Lanka	12.94	0.82	1.15	9.63	2.82	75.45
Sri Lanka Red Rice 2	Sri Lanka	11.12	1.12	2.19	9.52	2.87	76.05
Sri Lanka Red Rice 3	Sri Lanka	9.85	0.98	1.17	8.72	2.88	79.27
China Red Rice	China	11.90	1.37	2.35	9.72	2.52	74.66

1.2. Rice wine processing

Foods are the basic survival needs for human being. All over the world, various methods have been used to process and to preserve foods since ancient times. Throughout history and around the world, human societies at every level of complexity discovered how to make fermented beverages from sugar sources available in their local habitats (McGovern et al., 2004). Fermentation is one of the oldest and widely used as food preservation methods in households, small and medium-sized food industries as well as in large enterprise. Fermented foods mostly preserved pleasant flavour, aroma, texture, enhanced nutritive values and keeping quality under ambient conditions. Alcoholic beverages, including beers, wine and spirits, are an essential type of indigenous fermented product. They play an important role in human spiritual and cultural life. Many kinds of indigenous alcoholic beverages are widely produced and consumed. Holzapfel, (2002) described fermented

foods as palatable and wholesome foods prepared from raw or cooked raw materials. Several classifications had been used to categorize the wide spectrum of fermented foods including the diversification of microorganisms, different food groups and types of fermentation involved (Campbell-Platt and Fox, 1987; Steinkraus, 1997; Wood, 1994). According to Nout and Sarkar, 1999, fermented foods are typically unique and vary according to regions due to the variation in climate, social patterns, consumption practices and most importantly the availability of raw materials. Availability of raw materials brings about the conversion of the raw materials to different form of fermented food products in order to increase the food varieties as well as to maintain food security. Over years, fermentation techniques have gained huge importance due to their economic and environmental advantage (Subramaniyam, R. and Vimala, 2012) especially alcoholic fermentation. Alcoholic fermentation was divided into three categories including distilled spirit (brandy, whiskey and vodka), brewing alcohol beverage (beer, wine, sake) and last mixed of brewing with distilled spirit (liquor). Fermentation of cereal grains to produce a wide variety of foods had been a practice for long time. Rice wine is a popular alcoholic beverage in the Asian country. In most of the Asian countries, rice is fermented into alcoholic beverage by using mixed-culture(s) (Tamang et al., 2010) whereas in Europe, America, and Australia, most cereals like wheat, rye, barley and maize are fermented by natural fermentation or by adding commercial baker's yeast (Guyot, 2010). Eight countries in Southeast Asia produce paddy including: Indonesia, Thailand, Vietnam, Burma, Philippines, Cambodia, Laos, and Malaysia. These countries produce 150 million tons of paddy per year (25% of world production), of which 95% is consumed within the region (Mutert and Fairhurst, 2004). In Cambodia, Vietnam, Philippine, Thailand, Malaysia, China, Korea, the traditional alcoholic beverages made from rice starch are very popular and produced locally in large amount (Kofi E Aidoo et al., 2006; Chim et al., 2015b; Dung et al., 2007). This beverage has not yet benefited from scientific research and development efforts; however, it is particularly interesting because of its sherry-

like taste and fruity flavour, and its attractive brown-red colour. Its production is a source of income for farmer families in rural regions. It is produced under uncontrolled conditions at home-scale, using traditional solid-state ferment starters in tablet form. This practice leads the rice fermented beverage into low yields and variable quality. Whereas wine producers are aware that the use of ferment starter tablets influences the corresponding yield and quality of wine, there is very limited knowledge about the relation between the way they are prepared, their microbiological composition and their performance as starters. Ferment starter, similarly to koji used in sake brewing, is used for liquefaction and saccharification of rice starch. It has been reported that molds present in the starters produce starch-hydrolyzing enzymes, whereas yeasts from the same starters convert the sugars into alcohol (Nout and Aidoo, 2002). Various microorganisms are involved during the rice winemaking process and their growth and metabolism can have a considerable impact on the quality of a wine. Wine can therefore be described as the product of a complex interaction between different microorganisms, chemical constituents of raw material used and fermentation process for wine making. This fermentation is a complex process, involving a concerted series of microbiological, biochemical, and chemical reactions. The degradation of rice components by glycolysis, lipolysis, and proteolysis draws to the formation of a wide range of precursors of flavour compounds (Shou et al., 2007). These reactions are then followed and/or overlapped by a series of secondary catabolic reactions, for example, transformation of the free amino acids and fatty acids into important volatile compounds such as methyl ketones, alcohols, esters, aldehydes, lactones, and sulphur compounds which are responsible for the flavour characteristics of a variety of fermented food product (Ordonez et al., 1999; Marilley and Casey, 2004). The manufacture of rice wine can be characterized as a biological process whereby rice is converted into wine by physical, microbiological and biochemical operations, including steaming, inoculation with starter, mashing and fermentation. The general summary of traditional rice wine production processes is

shown in **Figure1-1**. In Cambodia, rice wines are produced predominantly at artisanal home- or cottage-level. Though each producer has his own way for making wine, depending on his individual experience and regional available raw materials, in principle, all producers use the same process. Powdered starch-based starter (about 1%-2% of the raw starchy materials) is mixed with steamed or cooked gelatinized rice, which is then incubated under ambient conditions (Chim et al., 2015a; Dung, 2013). There are two main stages of traditional fermentation process: solid-state fermentation and submerged alcoholic fermentation. After an initial period (2–3 days) of uncontrolled aerobic solid-state fermentation, then molds mash is mixed with water and allowed to undergo for further 3–4 days of a submerged alcoholic fermentation. The principle of rice wine manufacturing consists of the saccharification of steamed rice starch by fungal enzymes under aerobic solid-state fermentation and the mould mash is mixed with water and is allowed to submerged alcoholic fermentation by yeasts using traditional ferment starters (Blandino et al., 2003; Dung et al., 2007; Sujaya et al., 2004). Yeast is responsible for the alcoholic fermentation process during which available sugar are converted into ethanol and carbon dioxide. This process is mostly dominated by *Saccharomyces cerevisiae* yeast, but non-*Saccharomyces* yeast genera also associated with winemaking include *Candida*, *Cryptococcus*, *Debarymyces*, *Hanseniaspora*, *Kluyveromyces*, *Metschnikowia*, *Pichia*, *Rhodotorula*, *Saccharomycodes*, *Schzosaccharomyces*, *Torulaspora* and *Zygosaccharomyces* (Pretorius et al., 1999). Yeast can have an effect on wine aroma, flavour, mouth-feel, color and chemical composition by the production and excretion of metabolite during growth and autolysis (Swiegers and Pretorius, 2005). The most typical characteristic of rice wine fermentation is that this process is simultaneously saccharifying and fermenting (Shen et al., 2012). Rice-wine is a generic name referring to alcoholic beverages made from cereals, mainly rice. Similarly, Cambodian people apply the traditional fermentation beverage process to many raw materials such as non-sticky rice, glutinous rice, pigmented rice, cassava and other starchy resources. Traditional

alcoholic beverages range from crystal-clear products to turbid liquid or thick gruels and pastes. Clear products, which are generally called *Shaosingjiu* in China, *Cheongju* in Korea, and *Sake* in Japan, contain around 15% alcohol and are designated as rice-wine, whereas turbid beverages, takju (or maggolli) in Korea and Tapuy in the Philippines, contain less than 8% alcohol along with suspended insoluble solids and live yeasts, and are referred to as rice-beer (Rhee et al., 2011). Moreover, as most fields of research are focused primarily on *S. cerevisiae*, combination for synthetic communities for rice wine production with *S. cerevisiae* can benefit from the techniques developed by *S. cerevisiae* researchers.

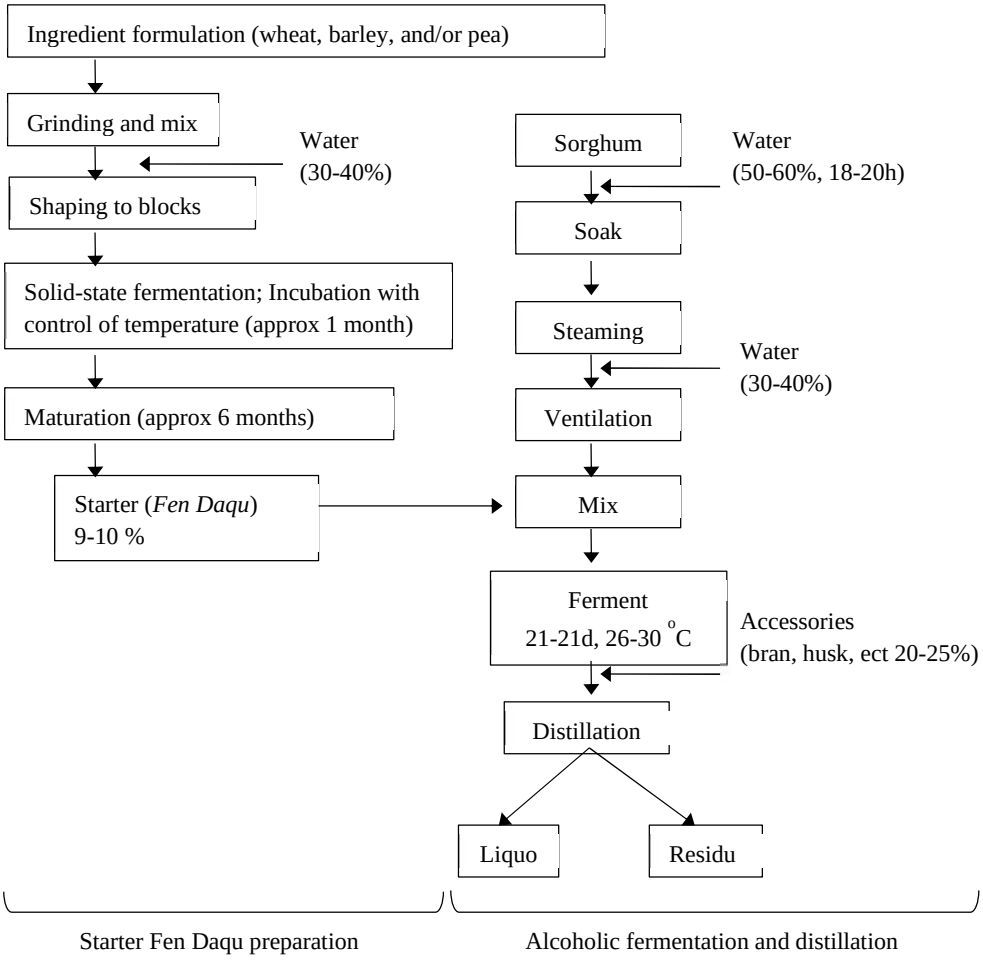

Figure 1-1: Process diagram for the production of fen-daqu and its role in the production process of Chinese liquor (Zheng et al., 2012).

1.3. Traditional ferment starter for red rice wine fermentation

Ferment starters are commonly used for starchy-base production in most of Asian country. The traditional home-scale technologies to produce ferment starter can vary according to the different regions. Ferment starter in tablet form is mainly a culture of

molds, yeast and bacteria grown on and within ground rice dough (Hesseltine et al., 1985). There are several starter cultures available in the markets in most Asian countries as summarized in **Table1-2**. They are prepared under non-sterile conditions by producers lacking microbiological training. In the preparation of starter cakes, rice or wheat is ground and thoroughly mixed with spices which are believed to play a major role in preventing growth of undesirable micro-organisms. A mixture of garlic, pepper, herb, rhizomes, onion and root is used in the preparation of the starter and producers regard their recipes as secret passed from generation to generation. Water is added to make a dough-like material which is shaped into small balls or flat cakes. Dry powdered starter from previous batches is sprinkled over the cakes. The latter are then placed on a bamboo tray, covered with a cloth and incubated at ambient temperature for 2-5 days during which the dough rises slightly and will be covered with fungal mycelia. The cakes are air- or sun-dried and have a shelf life of several months. To control microbial activities of the mixed cultures contained in starter cakes, pure cultures of the selected strains of *Rhizopus* spp. (mainly *R. oryzae*) have been used for industrial production of enzyme starters, in particular, for the distillery industry, and mycelial fungi and yeasts with amylolytic activities have been studied (Nout and Aidoo, 2002). The preparation and the use of fermentation starters as a source of inoculum are important in the manufacture of rice wine. It is recognized by winemakers that the choice of starter tablets influences the yield and quality of wine. Each rice wine producer in Cambodia, they have their own way of starter production depending on available ingredients; however, the products in principle must contain a combination of micro-organisms able to perform the microbiological and biochemical reactions that convert rice starch into wine (Chim et al., 2015b; Ly et al., 2018). The intention of adding oriental herbs in the traditional starter preparation has been studied with respect to their effect on the microbiota of starter during the manufacture. It was observed that 33 of 35 investigated oriental herbs stimulate the growth of yeasts and molds. There appeared to be a synergistic effect. A complex mixture of 10

medicaments is applied in practice and is effective and economic. The raw ingredients can be either rice flour or cassava flour or combinations of rice flour and cassava flour; however, the mixed flours are preferred by local producers. Rice wine ferment starter is known by several names, such as *men* in Vietnam, *paeng* in Laos, *look paeng* in Thailand, *marcha* or *murcha* in the Himalayan regions of India, *ragi* in Indonesia, and *bubod* in the Philippines, *Fen Daqu* in China. Local names for fermentation starters in Cambodia are *Dombae* or *Mae sra* (meaning a liquor starter) (Yamamoto and Matsumoto, 2011). The limited knowledge about traditional starters cause an obstacle to industrial development, and thus, these starters attract attention of researchers in food microbiology and technology. This has resulted in studies concerning the selection of safe, storable superior starters for small-scale fermentation processes and provide consistent and good quality to rice wine. Single, mixed or multiple-strain cultures have been investigated in Vietnam to select compatible strains and good combinations of complementary functionality (Dung et al., 2005). These were examined for viability when processed and stored in simple starch-based starters (Cronk et al., 1977; Djien, 1972; Hesseltine et al., 1985). Advantages of defined mixed starter cultures have been mentioned (Holzapfel, 1997; Ndip et al., 2001; Siebenhandl et al., 2001), and include: enhanced yield, improved hygiene, predictability of fermentation processes, and control of safety and quality. Given the biodiversity of wine ferment starter in regard to their production levels of enzymatic activities (Manzanares et al., 2000; Viana et al., 2008) and fermentation metabolites (Capece et al., 2005; Romano et al., 2003) of oenological importance, suitable strains should be selected and mixed in order to design mixed starters capable of providing beneficial contributions to wine quality. Consequently, having more information about the yeast communities existing in fermentation starters is important for wine-makers to produce wines with high quality and representative attributes. Until now, only a few studies have been conducted to describe the yeast diversity of Chinese rice wine fermentation starters (Shi et al., 2009; G. Xie et al., 2007) and very little or lack of research on

microbiota diversities of ferment starters which are being used in Cambodia. Research have been focused more on fermentation characteristics of some strains and the technological aspects of the wine production process. Systematic study about the yeast community of Hong Qu glutinous rice wine starters is essential to improve the wine-making practice and to guarantee the wine with flavour of regional attributes (Lv et al., 2012b; X.-C. Lv et al., 2013; X.-C. C. Lv et al., 2015; X. C. Lv et al., 2015). Ferment starters were prepared by various traditional method using mixed culture from microorganism present in the natural environment, equipment, substrate through the repeated used of the inocula originating from a previous fermentation or the previously prepared starter mother culture. The tablets of starters are generally shaped into small flattened or ball-shaped cakes about 4 cm in diameter and 1cm thick. The cakes are placed on a bamboo tray and are then covered with a thin layer of rice husks. According to producers this reduces overheating and facilitates aeration. The tray is covered with a cloth and incubated in a ventilated place at ambient temperature for 2-5 days during which time the dough rises slightly and becomes covered with fungal mycelium. The cakes are sun- or air-dried and then have a shelf life of several months (Dung, 2013). The traditional process of starter production in Vietnam is summarized in **Figure 1-2**.

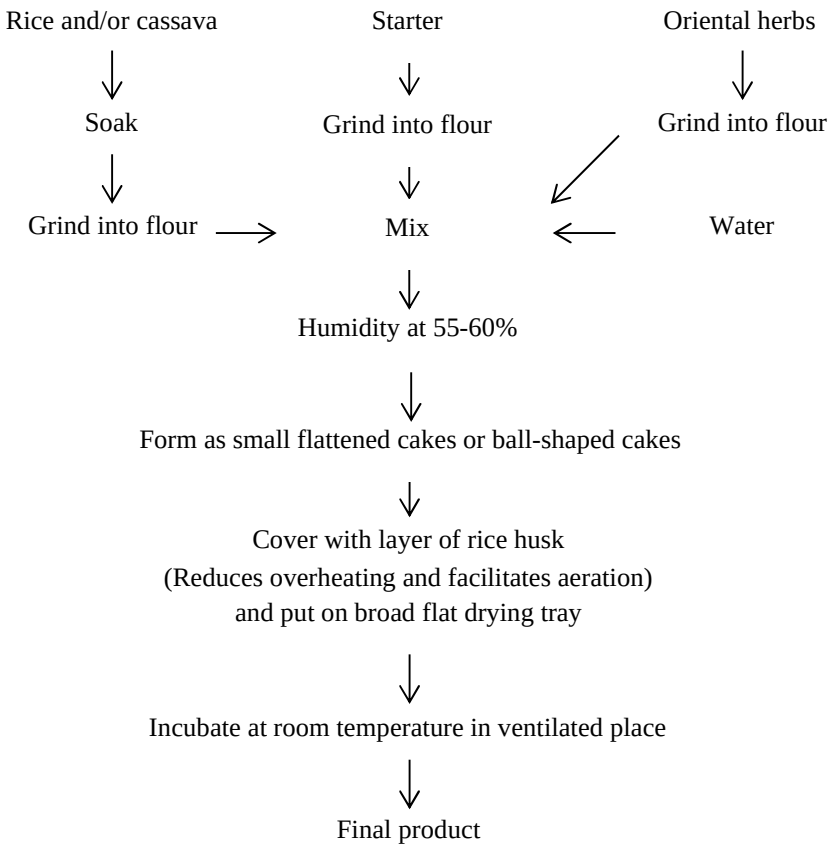

Figure 1-2: Traditional process of preparing rice wine starter (men) in Vietnam (Dung, 2013)

In India, there is also the production of traditional starter called "*Hamei*" which its production process is showed in **Figure 1-3**.

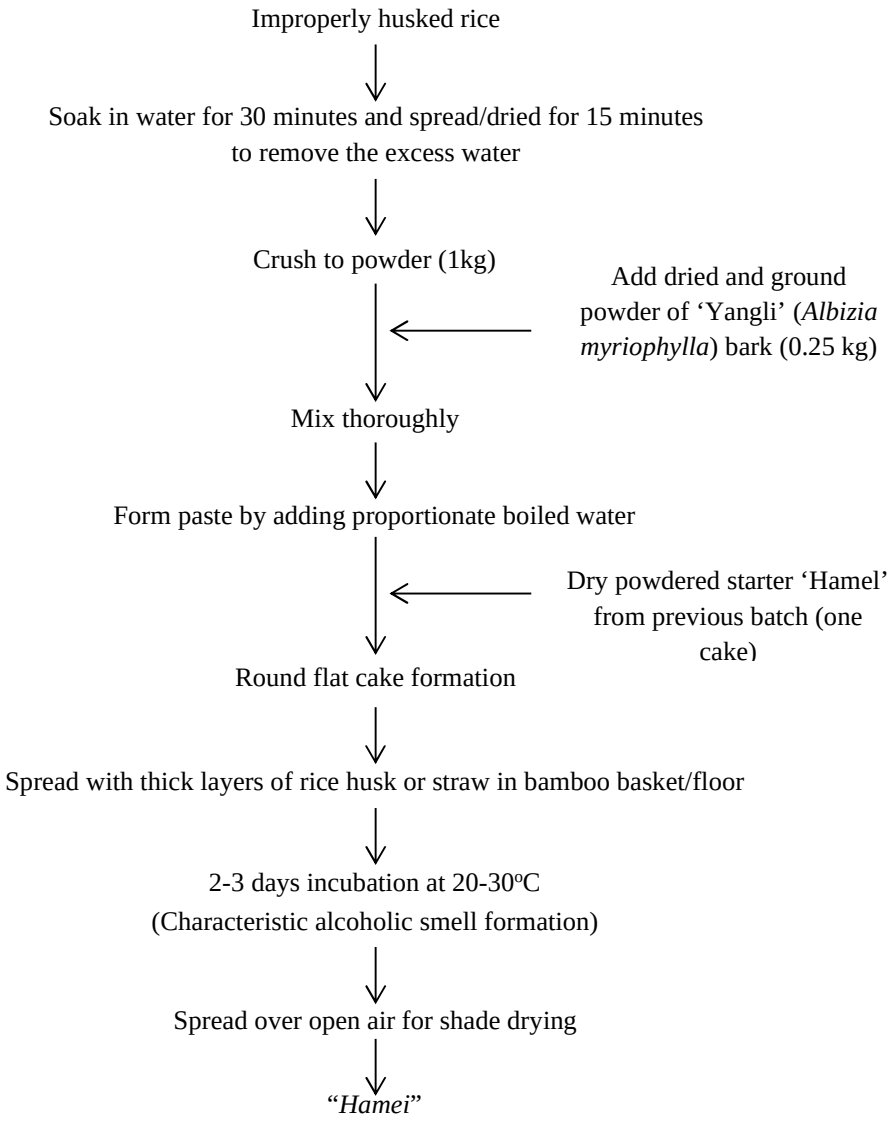

Figure 1-3: Process flow for traditional "hamei" in India (Jeyaram et al., 2008a)

Table 1-2: Microbial composition of starter culture for traditional fermented rice-based beverages in Asian countries

Product	Raw material	Functional yeasts and molds
Brem Bali, Tuak, Ciu	Rice, glutinous rice, sap of palm trees, cane-sugar	*Amylomyces* spp., *Mucor* spp., *Rhizopus* spp., *Candida* spp., *Saccharomyces* spp.
Bubod, , Lambanog, Tuba, Tapoi, Tapuy	Rice, roselle fruit, palmyra juice	*Aspergillus* spp., *Endomycopsis* spp., *Hansenula* spp., *Endomycopsis fibuliger, Rhodotorula glutinis, Debaromyces hansenii, Candida parapsilosis, Trichosporon fennicum, Saccharomyces ellipsoideus.*
Bupju, Takju, Yakju	Rice, glutinous rice, barley, wheat, millet	*Aspergillus oryzae, Aspergillus sojae, Mucor* spp., *Rhizopus* spp., *Saccharomyces cerevisiae, Hansenula anomala, H. subpelliculosa, Torulopsis sake, T. inconspicua, Pichia polymorpha*
Fenni, Sonti, Ruhi, Madhu, Jnard	Rice, Cashew apple	*Mucor, Rhizopus Aspergillus oryzae, Rhizopus spp., Saccharomyces cerevisiae*
Mie-chiu, Shaohing	Rice, wheat, barley	*Aspergillus oryzae, Aspergillus awamorii, Saccharomyces sake, Hansenula anomala*
Mirin, Sake, Shochu, Umeshu	Rice, maize, barley, plum	*Mucor* spp., *Rhizopus* spp., *Aspergillus* spp., *Saccharomyces ellipsoideus, Saccharomyces cerevisiae, Endomycopsis fibuliger, Hansenula anomala, Torulopsis candida*
Ruou De, Ruou Nep, Ruou Nep Than, Ruou Can, Ruou Vang	Rice, (purple) glutinous rice, maize, cassava	*Mucor* spp., *Rhizopus* spp., *Candida* spp., *Saccharomyces* spp.
Sato, Ou, Nam-Khao Tapai, Samsu	Rice, glutinous rice	*Amylomyces rouxii, Rhizopus* spp., *Endomycopsis* spp.
Dombea, Me sra	Rice, (purple) glutinous rice	*Rhizopus* spp., *Saccharomyces* spp., *Saccharomycopsis* spp.

* Based on data of Hesseltine, 1983; Luong, 1998; Haard et al., 1999; Nout and Aidoo, 2002.

In Cambodia, rice wine is fermented from glutinous rice or non-sticky rice with *Dombea* under spontaneous conditions, and it can be rich in various microorganisms and complex enzyme during fermentation. Therefore, *Dombea* plays a key role in Cambodian rice wine production. It is not only a source of enzymes essential for the breakdown of carbohydrates and proteins in rice grain, a supplier of varieties of microorganisms, but also a source of flavour and aroma compounds in rice wine. During rice wine making using *Dombea*, various microorganism, various enzyme, and different substances present in the mash of wine. So, the characteristics of *Dombea* directly influence the quality of rice wine. Some research have focused on enzyme and microorganism in ferment starter and volatile compounds (Chen et al., 2013; Dung et al., 2005; Ghosh et al., 2015; Sirisantimethakom et al., 2008; Wang et al., 2014b, 2014a), yet lack of research in Cambodia.

1.4. Microbial interaction in view of flavour compound

As far as consumers are concerned, the aroma and flavour of wine are among the main characteristics that determine its quality and value (Swiegers et al., 2005). Aromatic compounds were mainly formed through aromatic amino acids metabolism (Diaz et al., 2001). Volatile compounds play an important role for the quality of wine. More than 800 volatiles have been identified in wines including alcohols, esters, organic acids, aldehydes, ketones and monoterpenes (Howard et al., 2005). The concentration of these compounds in wines varies from little mg L^{-1} to hundreds of mg L^{-1}. The aroma complexity productively increases during alcoholic fermentation as a result of the synthesis of important volatile compounds by the wine yeast *Saccharomyces cerevisiae* and the release of some varietal aroma precursors (Swiegers et al., 2005). The nature and amount of the synthesized volatile compounds depend on multiple factors, such as the nitrogen content of the must, the temperature of fermentation and

the yeast strain (Lambrechts and Pretorius, 2000; Swiegers et al., 2006). The volatile compounds synthesized by wine yeasts include higher alcohols (fusel, marzipan and floral aromas), medium- and long-chain volatile acids (fatty, cheesy and sweaty aromas), acetate esters and ethyl esters (fruity and floral aromas) and aldehydes (buttery, fruity and nutty aromas), among others (Delfini et al., 2001; Lambrechts and Pretorius, 2000; Stashenko et al., 1992). Higher alcohols can be synthesized either from intermediates of sugar metabolism, through anabolic reactions, or from branched-chain amino acids, through a multi-step catabolic reaction, the Ehrlich pathway (Dickinson et al., 2003, 1997; Eden et al., 2001). The volatile fatty acids also contribute to the aroma of wines. Fatty acids are essential constituents of the plasma membrane and precursors of more complex molecules, such as phospholipids. During the fermentation and storage period, change of enzymes, microorganisms, and substances occur and the change depend on many factors including temperature, humidity, and time of fermentation and storage (Wang et al., 2005). The concentration of its volatile compounds is present in trace amounts; therefore, it is necessary to have a sample preparation technique e.g. Solid Phase MicroExtraction (SPME) or Dynamic head-space (DHS) technique. These methods are solvent-free extraction technique and rapid sample preparation. These methods can extract a wide range of aroma compounds and have been widely use for the analysis of volatile compounds. Most of these compounds are produced during must fermentation and are especially important in the aroma of young wines. Acetic acid, acetaldehyde, ethyl acetate, propanol, isobutanol, 2- and 3-methylbutanol account for more than half of these volatiles, the other half being distributed among 600–800 minor volatile compounds present in very low amounts (acetals, organic acids, alcohols, phenolic and heterocyclic compounds, esters, lactones, terpenes and sulfur-containing compounds). The analysis of the contribution to the aroma of these minor compounds is complicated because of their low concentrations and their interactions. The quantity and quality of the aromas and flavours originated in must fermentation depend on environmental conditions,

vilification process and the participating yeasts. For grape wine, the alcoholic fermentation (AF) (primary fermentation) carried out by yeast (often separated into *Saccharomyces* and non-*Saccharomyces* species generally followed by malolactic fermentation (MLF) (secondary fermentation) conducted by bacteria. MLF is conducted by lactic acid bacteria (LAB), mainly represented by species of *Oenococcus*, *Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Weissella* (Bae et al., 2006; Holzapfel et al., 2009; Lonvaud-Funel, 1999). Even though malic acid conversion can be conducted by any of the wine associated bacteria, *O. oeni* is the main species associated with MLF because of its tolerance to the harsh wine conditions (such as high ethanol concentration, low pH and nutrient content)

(Ribéreau-Gayon et al., 2006). The onset of MLF can be controlled through the inoculation of a selected *O. oeni* or *L. plantarum* strain, however, under favorable conditions, MLF can occur spontaneously. The growth of the indigenous wine LAB population is related to their diverse ability to utilize sugars, amino acids and to tolerate multiple stresses, including acidity, SO2, high concentrations of ethanol, and low temperatures (Borneman et al., 2012a; Ribéreau-Gayon et al., 2006).

Microorganisms are rarely encountered as single species populations in the environment, since studies in different habitats has shown that an enormous richness and abundance variation are usually detected in a small sample, suggesting that microbial interactions are inherent to the establishment of populations in the environment, which includes soil, sediment, animal, and plants, including also fungi and protozoa cells. Many secondary metabolites have been reported to be involved in the microbial interactions. These compounds are usually bioactive and can perform important functions in ecological interactions. A widely studied mechanism of microbial interaction is quorum sensing, which consists in a stimuli-response system related to cellular concentration. The characteristics of any spontaneously fermented product are a composite result of the metabolism of a variety of microorganisms. More

complicated is the interaction of complementary metabolisms, where a compound produced by one organism may be metabolized further by another. A good example of this is found in DL mesophilic lactic starters where the strains of *Leuconostoc* present reduce acetaldehyde produced by the *Lactococcus* strains

(Tamime and Marshall, 1997).

Fungi can colonize surfaces of a wide range of raw material, however; in submerged culture processes, they typically exhibit a complex morphological life cycle that is related to production performance – a link that is of high interest for process optimization. The fungal forms can vary from dense spherical pellets to viscous mycelia. The resulting morphology has been shown to be influenced strongly by process parameters, including power input through stirring and aeration, mass transfer characteristics, pH value, osmolality and the presence of solid micro-particles. Due to their metabolic diversity, high production capacity, secretion efficiency, and capability of carrying out post-translational modifications, filamentous fungi are widely exploited as efficient cell-factories in the production of metabolites, bioactive substances and native or heterologous proteins, respectively. The commercial use of fungal microorganisms has been reported for multiple industrial sectors, such as those involved in the production of simple organic compounds (e.g., detergents), food and beverages, or pharmaceuticals. Quantitative characterization, monitoring, and even defined control of the morphology of filamentous growing fungi are difficult to obtain, which is inherently linked to the highly complex morphological types and the bi-directional interaction between morphology and the process environment, e.g., via broth rheology or mixing and mass transfer performance. The morphological type and related physiology of fungal systems strongly depend on the environmental conditions in the bioreactor, i.e., the environment (Krull et al., 2010). Among other factors, the variable environmental cultivation conditions comprise the inoculum concentration, spore viability, pH value, temperature, dissolved oxygen concentration, and

mechanical stress (Deckwer et al., 2006). The effect of agitation on fungal morphology has been the subject of several studies (Cui et al., 1998; Fujita et al., 1994; Kelly et al., 2004).

SCIENTIFIC STRATEGY

Countless researches have focused on enzyme and microorganism in ferment starters and their impact on wine quality, yet very little or lack of research for rice wine fermentation technology development in Cambodia. The limited knowledge and research interest cause an obstacle to rice wine industrial development. In order to mitigate the missing information, this work is based on the analysis and control of microbial communities implied in the fermentation of red rice wine. After the analysis of microbial communities by amplicon sequencing, major microbial species have been selected in order to build up synthetic communities. These strains have been selected in function of their ability to metabolize the main carbon and nitrogen sources and also to their impact on volatolomic profile of the end product. In our case, these synthetic communities comprise a set of microbes able to platform a cascade of biochemical reactor leading to the final product, i.e. fermented red rice wine. This present thesis addresses the problem of poor and variable quality of rice wine production in Cambodia through the understanding of the impact of microflora in presented in ferment dried starters (*Dombea*) on the quality of rice wine. For preliminary field work, the processes of making ferment starter and rice wine were observed, and five represent ferment starters samples were collected from local producers. **Chapter 3.1** described about the impact of microbial composition of those five fermentation starters on flavour compounds of rice wine by combining amplicon sequencing and SPME-GCMS technique. After the analysis of microbial communities by amplicon sequencing, major microbial strains have been selected in order to build up synthetic communities. These strains have been selected in function of their ability to metabolize, their important role during fermentation and according also to their impact on the volatolomic profile of the end product. Then, the exploitation of microbial interactions in bioprocesses through the use of synthetic communities and biofilm packing system was studied and discussed in **Chapter 3.2**. In order to develop

and control the system to improve the performance of filamentous fungi, **Chapter 3.3** focused on the performance as well as the evolution of fungi on inert support material that can be easily transferred for setting-up more effective biofilm reactors.

2

BIBLIOGRAPHY

2.1. Wine fermentation: European and Cambodian processes

2.1.1. Grape wine production and its quality

Grapes are one of the most well-known and pleasant fruits in the world. Evidence has shown that raisin was produced by the Egyptians as early as 6000 B.C. Raisin specifically have been mentioned in ancient writing and it suggests that they were used for eating, treating illnesses and even paying taxes. Over the years, wine making has been the most important use for grapes. The fruit of the grapevine provides excellent substrate for fermentation and the ideal surface of the grape berry is a favourable habitat for yeasts. It is probable that wine was the unexpected result of the storage of grape during the winter period. With the development of village settlements, it is likely that the best of the wild vines from the forest were brought into cultivation and thus began the history of viticulture and oenology (Mullins et al., 1992). Various microorganisms are involved during the winemaking process and their growth and metabolism can have an immense impact on the quality of a wine. Wine can therefore described as the product of a complex interaction between different microorganisms and chemical constituents of raw material used for wine making (Fleet, 2003). The limited number of commercially available yeast strains, used for global wine-making production, is thought to contribute to production of wines with relatively uniform style compromising a diversity and wine potential. The process starts from the vineyard, continues through fermentation and maturation, and concludes at packaging. It is affected by the various viticultural and oenological practices available to the grape-grower and winemaker, respectively (Regueiro et al., 1993). All of microorganisms involved, yeasts are the heart of biochemical interaction, they conduct the alcoholic fermentation (conversion of fermentable sugar in grape must to ethanol and CO_2). Quality control during the winemaking process ensures that the final product is competitive on the international market and of a

sufficient quality standard. The quality of wine is usually evaluated on its visual, olfactory and taste characteristic. Moreover, although wine flavours is directly derived from grape variety, yeasts also affect wine flavour and quality by the production and excretion of metabolites during growth and through autolysis (Fleet, 2003; Lambrechts and Pretorius, 2000; Swiegers et al., 2005; Swiegers and Pretorius, 2005). In wine production, natural grape juice fermentation is carried out by a succession of different yeast populations. Yeast can have an effect on wine aroma, flavour, mouth-feel, colour and chemical composition by the production and excretion of metabolite during growth and autolysis (Swiegers et al., 2005). Yeasts present during fermentation are derived from grapes and the vineyard, the equipment used in the cellar, cellar surfaces and external sources such as selected cultures that are added to facilitate the fermentation process. *S. cerevisiae* produces most of the ethanol in wine, non-*Saccharomyces* yeasts (NS) present, play a significant role in producing aroma compounds, such as esters, higher alcohols, acids and monoterpenes contributing with diversity of flavour phenotypes (P Romano et al., 1997; Swiegers and Pretorius, 2005). As the fermentation progresses, the non-*Saccharomyces* species successively die off (Heard and Fleet, 1988; Longo et al., 1991; P Romano et al., 1997), leaving S. *cerevisiae* to dominate and complete the fermentation. Non-*Saccharomyces* yeast genera associated with winemaking are *Candida*, *Cryptococcus*, *Debarymyces*, *Hanseniaspora*, *Kluyveromyces*, *Metschnikowia*, *Pichia*, *Rhodotorula*, *Saccharomycodes*, *Schzosaccharomyces*, *Torulaspora* and *Zygosaccharomyces* (Pretorius et al., 2017). The early stages of the alcoholic fermentation are generally dominated by yeasts belonging to the genus *Hanseniaspora* (*Kloeckera*), as well as by other non-*Saccharomyces* yeasts like *Candida stellata* and *Metschnikowia pulcherrima* (Fleet, 2003). *Saccharomyces* spp. was never detected in fresh must samples plated on non-selective YEPD media, so its initial presence should have been in much lower numbers than the non-*Saccharomyces* species. The grape must with its chemical properties (low pH, sugars contents, the rising amount of ethanol during the

must fermentation) is a effective preferential medium, especially appropriate for the *S. cerevisiae* yeast, and it enables this species to dominate in all phase of fermentation. The diversity of yeast species correlates with the formation of secondary products of fermentation, such as acetaldehyde, ethyl acetate and higher alcohols (Ciani and Picciotti, 1995; P Romano et al., 1997; Patrizia Romano et al., 1997). Grape must is a rich nutritive environment, but low pH and high osmotic pressure of the must and the use of SO_2 detracts from this otherwise ideal yeast niche. Surfaces of cellar equipment can also harbour numerous microorganism due to constant contact with grape must. The non-*Saccharomyces* species proliferate from an initial population of 10^4 to 10^5 cells ml^{-1} in the grape must to the final populations of approximately 10^6 to 10^8 cells/ml. Despite the fact that there is a succession of yeast genera and species involved in the spontaneous fermentation, only a few *Saccharomyces cerevisiae* strains dominate the fermentation. Given its importance in product quality, much effort has been devoted to fine flavour production by yeast in an industrial setting. Globally, two approaches can be applied to steer the yeast's physiology to alter aroma production: adjusting the fermentation environment or modifying the genotype of the production strain. Adjusting the environmental parameters can be a convenient, often very powerful, way to optimize production without complex biotechnological procedures nor a thorough understanding of basic yeast physiology. However, given the recent expansion of the available yeast biodiversity, strategies to modify yeasts and the genetic toolbox to genetically engineer strains, biotechnologists can now select or develop new yeasts with aromatic properties far beyond what is achievable through adjustment of environmental parameters.

Malolactic fermentation (MLF), the enzymatic decarboxylation of L-malic acid to L-lactic acid, is an important secondary fermentation carried out by lactic acid bacteria (LAB) during the vinification. In addition to deacidification, MLF can increase microbiological stability and enhance the flavour and aroma of wine (Bae et al., 2006; Beelman and Gallander, 1979; Davis et al., 1985). Traditional practices have relied

upon the growth of naturally occurring LAB microflora to induce MLF spontaneously. However, the harsh physico-chemical conditions of wine, including low pH, high ethanol content, presence of sulphur dioxide and low nutritional status, create a stressful environment for the growth of ML bacteria (Wibowo et al., 1988). In these conditions, failure of MLF to develop is not an uncommon experience. To overcome these difficulties, continued developments in ML strain selection and starter culture technologies have facilitated greater control over MLF induction.

2.1.1. Rice wine production in Asian country

In most of the Asian countries, rice is fermented either by using mixed-culture(s) into alcoholic beverages or by using food beverages (Tamang et al., 2010), whereas in Europe, America, and Australia, most cereals like wheat, rye, barley and maize are fermented by natural fermentation or by adding commercial baker's yeast into the batter for dough breads/loaves (Guyot, 2012). In Africa, fermented cereal foods are traditionally used as staples as well as complementary and weaning foods for infants and young people (Tou et al., 2007). As rice is the primary staple diets, it comes as no surprise that the rice-based alcoholic beverage is a potential brew made product. Rice-based traditional alcohol beverages are popular in Asian countries, such as Japan (sake), China (jiu), Korea (yakju), Philippines (tapuy), Vietnam (ruou nep than), Malaysia (tapai), Cambodia (tapae), Malaysia and Indonesia (tapai), and Thailand (sato) (Bakar et al., 2011; Kim et al., 2004; Kotaka et al., 2008; Luangkhlaypho et al., 2014; Ly et al., 2018; Thanh et al., 2008; Wang and Zhang, 2013).

In rice wine fermentation, solid-state fermentation is the early stage for molds to hydrolyse starch into smaller saccharide as substrate for yeast. In this stage, facultative aerobic and solid state of steamed rice with moisture content from 60-65% are the favor condition for mold to grow (Dung et al., 2007; Ly et al., 2018). The main alcoholic fermentation occurs during the second stage after water has been added and lasts for approximately 5 days. During this fermentation stage, yeast plays an

important role to convert fermentable sugar to ethanol, carbon dioxide and various kinds of fermentation by-products such as organic acids and flavour compounds (Srithamma, 2009). Yeast cells afterward start to consume glucose and produce ethanol more rapidly due to the better contact of enzymes with substrate. However, ethanol is not the only product from glucose conversion of yeast. The alcoholic fermentation (AF) is the main activity by which yeasts make a positive contribution to wine flavour. This stage is where organic acids, amino acids, CO_2, fusel alcohol, and many hundreds of flavour active, secondary metabolites (e.g. acids, alcohols, esters, polyols, aldehydes, ketones, volatile sulphur compounds), are produced through the Erhlic pathway (Fleet, 2003). The AF span varies depending on the amount of yeast and substrate, usually ended in 4 to 10 days fermentation. Lv and his team revealed that at early days of fermentation of AF, diverse groups of microorganisms can grow in present of water and rich in nutrient substrate, but three days afterward, the bacterial community is decreased and only the high ethanol tolerance strain such as *L. plantarum* was found in the rice wine (X.-C. Lv et al., 2013). This study indicates that AF is a stage to eliminate pathogenic microorganisms in ferment products. In contrast, if the growth of microorganism besides starter surpass the growth of the starter, it can affect the production yield and lead to wine spoilage. The simultaneous fermentation combines the saccharification step, alcoholic fermentation step, and lactic fermentation step together. Many studies on the simultaneous fermentation between mold and yeast (Dung *et al*., 2005; Zhang *et al*., 2007). The bioconversion of sugar to ethanol can be made much more effective by coupling the hydrolysis enzymes and ethanol fermentation of the derived sugar into a single step, called simultaneous saccharification and fermentation (SF). SF gives a decrease in the inhibition caused by minor disaccharide accumulation, leading to an increase in the saccharification rate, consequently increasing productivity and reducing reactor volume and capital cost. Hence, this fermentation method gives benefit to big scale fermentation for its time and cost economical. However, a

disadvantage associated with SF is the difference in cultivation conditions such as temperature and pH required for saccharification versus fermentation. Therefore, characterization of the microbial and biochemical kinetics and determination of the optimal process conditions which enhances SF is of needed for optimal final ferment product (Zhang *et al.*, 2007). In contrast to SF of mold and yeast, there is a limit of research studies on simultaneous with lactic acid bacteria. There is a past study on engineered yeast strain that can perform alcoholic and lactic fermentations at the same time (Tilloy *et al*., 2015). Although this strategy from the study was efficient, a substantial reduction of ethanol yield had occurred due to the accumulation of high levels of lactate which contributes to total acidity and may render the wines out of balance. Therefore, there is a need for further studies on both simultaneous and separate fermentation of yeast and LAB as ferment starter. The moulds involved in alcohol production of Asian rice wines include *A. rouxii*, *Rhizopus* spp., *Aspergillus* spp., *Mucor* spp. (Aidoo et al., 2006; Dung et al., 2007; Swiegers et al., 2005) and the yeasts *S. cerevisiae*, *Sm. burtonii*, *Sm. fibuligera*, *Cm. lactosa* and related yeasts. Rice and/or other cereal-based wines are produced on both a cottage and a commercial scale in most Asian countries (Nout and Aidoo, 2002). The production of alcoholic beverages culturing traditionally with essential microorganism is remarkable discovery in the food history of Asian people which is exclusively practices in South-East Asia (Hesseltine et al., 1985; Tamang et al., 2010). To make the dried ferment starter, approximately 1–2% from previously prepared amylolytic starters are inoculated into the dough (mixture of soaked rice and/or starchy based material), then mix and incubate for a short time to grow, dried later, and used to make either alcohol or fermented foods from starchy materials (Tamang et al., 1996). Asian amylolytic starters have different vernacular names such as Marcha in India and Nepal; Hamei, Humao, Phab in India; Mana and Manapu of Nepal; Men in Vietnam; Ragi in Indonesia; Bubod in Philippines; Chiu/Chu in China and Taiwan; Loogpang in Thailand; Maedombea or Dombae in Cambodia; and Nuruk in Korea (Chim et al.,

2015b; Hesseltine, W; Kurtzman, 1990; Nikkuni et al., 1996; Sujaya et al., 2004; Thanh et al., 2008; Yamamoto and Matsumoto, 2011). Microbial profiles of amylolytic starters of India, Nepal, and Bhutan are filamentous fungi including *M. circinelloides*, *M. hiemalis*, *R. chinensis*, and *Rhizopus stolonifera*, *Absidia corymbifera*, *Amylomyces rouxii*, *Botryobasidium subcoronatum*, *Rhizopus oryzae*, *R microsporus*, *Xeromyces bisporus* (Tamang et al., 1988); yeasts like *Candida tropicalis*, *Clavispora lusitaniae*, *Pichia anomala*, *Pichia ranongensis*, *Saccharomycopsis fibuligera*, *Saccharomyces cerevisiae* and *Issatchenkia* spp (Chakrabarty et al., 2014; Jeyaram et al., 2008b; Shrestha et al., 2002; Tamang and Sarkar, 1995; Tsuyoshi et al., 2005) species of LAB namely *Pe. pentosaceus, L. plantarum, Weissella confusa, Weissella paramesenteroides*, *L. bifermentans*, and *L. brevis* (Chakrabarty et al., 2014; Dung et al., 2007, 2006; Tamang et al., 2007, 1988; Tamang and Sarkar, 1995; Thanh et al., 2008). The industrial brewing follows the Japanese saké brewing method and undergoes relatively high temperature fermentation (25^{o}C). The alcohol content in the industrial rice wine tends to be higher but the wine is lower in esters than the traditional brew. At the end of fermentation, the rice wine is a turbid suspension of pink red color, containing 8–14% (w/v) alcohol and some residual sugars. The wine may be clarified and/or strengthened by blending with distilled alcohol, depending on local demand. The Cambodian rice-wine, *Srasor*, is made from glutinous rice or non-glutinous rice mixed with a traditional solid-state starter called *Dombea*, which is composed of a mixture of various microorganisms grown on rice or rice flour. *Sraa Sor*, which is one of the most popular Cambodian traditional rice, generally involves with distillation process. Dombae or Mesra (rice cake starter culture or fermented rice cake is a traditional starter culture used to produce alcoholic or fermented foods in Cambodia, such as srasor (Khmer traditional rice liquor), tapae (fermented waxy rice), srapeang (fermented rice wine), seang (fermented soybean), teukkhmes (vinegar) (Chim et al., 2015; Yamamoto and Matsumoto, 2011).

2.2. Methods and approaches to quantify microbial interactions

2.2.1. Microbial interactions

Most microbial life takes place in complex multi-species microbial communities and dependent on metabolite-mediated microbe-microbe and microbe-environment interactions. Konopka (2009) defines the term “community” as multispecies assemblages, in which organisms live together in a contiguous environment and interact with each other. Microbial interactions are crucial for a successful establishment and maintenance of a microbial population. These interactions occur by the environmental recognition followed by transference of molecular and genetic information that include many mechanisms and classes of molecules. These mechanisms allow microorganisms to establish in a community, which depending on the multitrophic interaction could result in high diversity. Studying single species of bacteria axenically was essential for birth of modern biochemistry and molecular biology and remains important to this day. However, even as early as the 1870s microbiologists including Louis Pasteur reported phenomena resulting from interactions of bacteria existing in multispecies communities (Pasteur, 1877). Bacteria interact extensively within and between species while responding to external stimuli from their environments. Indeed, the ability to perceive neighbouring cells and the environment is often reflected in the content of bacterial genomes. Thus, understanding how the genome-encoded functionalities of individual species affect global community interactions and dynamics has important environmental and biomedical applications. Bacteria living within communities often have access to a wider range of nutrients than single cells living in isolation from other species (Ponomarova and Patil, 2015). Metabolic syntrophism suggests that cells cooperate because different species possess complementary biochemical pathways needed to liberate nutrients from the environment (Morris et al., 2013). Metabolic

interactions, leading to cooperation, exploitation, or competition, are ubiquitous in multispecies biofilm and play important roles in maintaining the diversity and stability of microbial communities (Embree et al., 2015; Møller et al., 1998; Zelezniak et al., 2015) secondary metabolites have been reported to be involved in the microbial interactions. These compounds are usually bioactive and perform as important functions in ecological interactions. Microbial interaction deserves attention from researchers who work on natural products discovery field. Some secondary metabolite clusters that are silent under laboratory growing conditions, can be activated by simulating in natural habitat. It has been revealed that co-cultivation with other microorganisms from the same ecosystem can activate silent biosynthetic pathways leading to the production and identification of new natural products (Brakhage and Schroeckh, 2011; Netzker et al., 2015). Soils microbial communities also play a major role in protecting plants from diseases and abiotic stresses (Degrune et al., 2015) or increasing nutrient uptake. Another example during the traditional fermentation progressed, some microbes with inherent function (such as species of *Lactobacillus* and *Saccharomyces*) might secrete abundant organic acids and ethanol to make a change of the culture environment, which killed most of the extrinsic microbes and presented a "system microbes self-domestication" function (Ponomarova et al., 2017). There is a large variety of types of microbial interactions concerning the organisms involved. Bacteria–bacteria, fungus–fungus, bacteria–fungus, fungus–plant/animal, bacteria–plant/animal and bacteria–fungus–plant/animal interactions have been described allowing to develop strategies to manipulate these interactions. Fungi and bacteria interactions are widely studied, although the molecular mechanisms involved in the interactions are often not completely understood. Microbiological tools and their potential use in co-cultivation studies have been deserved attention from researchers.

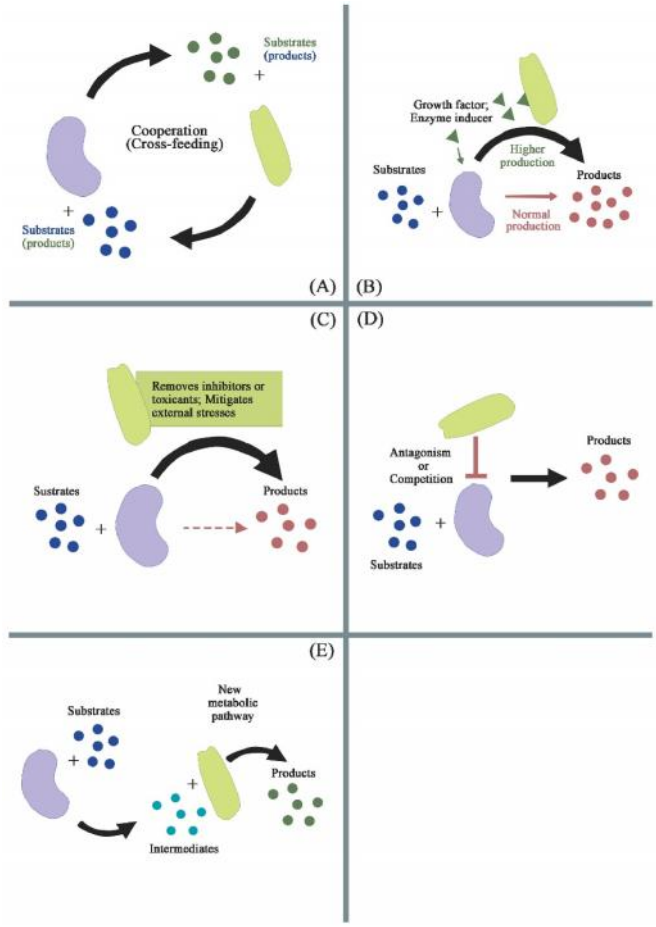

Figure 2-1: Schematic illustration of microbial consortia mechanisms.

Cells represent microbial species from different kingdoms, including archaea, bacteria, fungi and algae. A. Synergistic division of resources (e.g. Cross-feeding) and expansion of the resources spectrum (beneficial interactions). In general, cooperation within the microbial consortia can improve microbial metabolism, as considered in division of labour. B. Stimulated microbial growth and biotransformation. This commensal relationship is a type of symbiosis. C. Enhanced tolerance of inhibitors or toxicants to mitigate external stress and inhibition. This relationship can be achieved via inhibitor or toxicant removal by partner species. The dotted arrow represents decreased or inhibited product formation. D. Antagonistic interactions lead to production of beneficial metabolites and enzymes that may not be produced otherwise. |– Represents antagonistic or competitive interactions. E. Assembled biotransformation pathway to optimize efficiency and improve consortia robustness. (Zhang et al., 2018).

The exchange of diffusible metabolites and communication signals between consortia compared to monoculture and can significantly broaden the carbon and energy spectrum for each member (**Figure 2-1 A-B**).

Species residing in microbial communities with complex structures frequently affect the growth of the others by both cooperative and competitive interactions (Kim et al., 2008; Nadell et al., 2009), and Several challenges to constructing an artificial consortium can be mitigate by cooperative relationships (**Figure 2-1C**). Spatial organization has been demonstrated to play an important role in reducing the strength of between-species cooperative interactions, contributing to the stability of multispecies communities, such as gut microbiome (Coyte et al., 2015). However, once interspecific competition occurs when individual interact indirectly as they compete for common resources such as territory, prey or food. An antagonistic interaction can gain some production of beneficial metabolites and enzymes that may not be produced in monoculture **Figure 2-1D**. Interkingdom consortia could be proposed to design heterologous microbial functions to assemble novel biochemical pathways and generate unique products (**Figure 2-1B**). **Figure 2-2** gives the example of spatial interactions in microbial consortia. The interkingdom consortium maintains stability and strengthen against external stress by physical and spatial structure modifications (Flemming et al., 2016; Smith et al., 2015; Zhang et al., 2018) microbiota can physically interact via aggregation, surface charge, immobilization Consortia can physically interact via aggregation (e.g. chemotactic responses) (Seymour et al., 2010; Sourjik and Wingreen, 2012), surface charge (e.g. fungal–algal communities) (Wan et al., 2015) or immobilization (e.g. filamentous microorganisms acting as sorbents and sources of nutrients for the surface attachment of others) (Hogan, 2002).

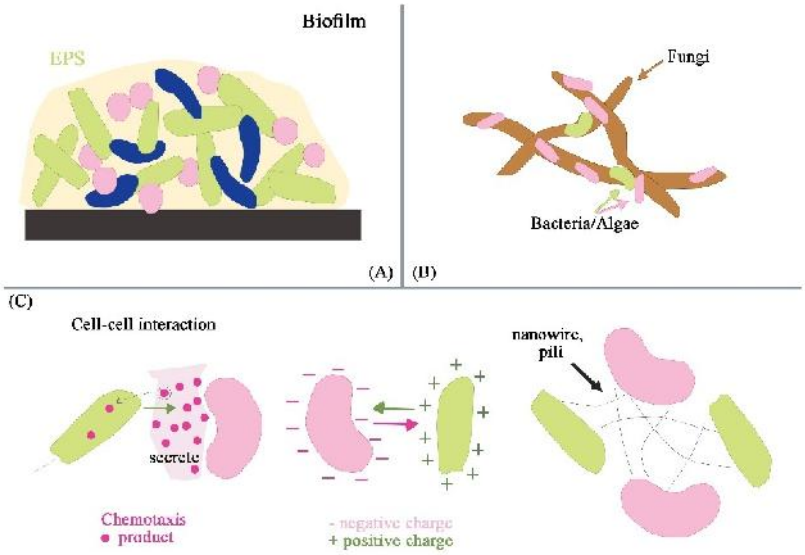

Figure 2-2: schematic depiction of spatial interactions in microbial consortia.

A. Biofilms are matrix-enclosed microbial populations co-localized to surfaces or interfaces and have been applied for bioprocessing and biotechnological purposes via artificial design. B. Symbiotic interaction among diverse cells including filamentous species (e.g. Fungi) and other microorganisms (e.g. Bacteria and algae) by surface attachment. C. Cell–cell interaction, including chemotaxis response induced by small diffusible molecules secreted by microorganisms; interactions related to positive and negative surface charges and attachment of nanowire/nanotubes between species to transport growth essentials and communication signals, such as electrons and protons. D. Endosymbiosis consists of one or more prokaryotic species living within a host cell. (Zhang et al., 2018).

2.2.2. Current microbiological tools and their potential use in co-cultivation

Microorganisms are everywhere, but they rarely act alone. It is now known that many microorganisms depend on the activity of other microorganisms to successfully grow and reproduce (Hug et al., 2012; McCutcheon and Von Dohlen, 2011; Schink, 2002; Stolyar et al., 2007) via mechanisms including acquisition and exchange of metabolites (Carini et al., 2013; Falony et al., 2006; Stams, 1994). Many microbial biosynthetic genes are apparently not transcribed under standard laboratory conditions but remain silent. Consequently, only a fraction of the real biosynthetic diversity of

microbes is obtained in terms of produced compounds. Several strategies have been applied trying to overcome these limitations during fermentation of microbes. These approaches introduce where promising strains are cultured in a variety of media and under different culture conditions in order to maximize the diversity of compounds produced (Bode et al., 2002). Species interactions in microbial communities can be either metabolism-based or be driven by ecological traits. Several good reviews have summarized the study of ecological interactions among microbes in synthetic as well as in natural microbial communities (Faust and Raes, 2012; Mitri and Richard Foster, 2013). Although species are commonly thought of as ecological units and thus the most natural entities to count and search for interactions, several reasons motivate the use of other units to quantify biodiversity. Larger taxonomic groups may be of interest because they share one or more important attributes: class amphibia is seen as a leading indicator of a general decline in biodiversity, in part due to their sensitivity to disturbances in both terrestrial and aquatic habitats, while Bacteroidetes and firmicutes are sometimes treated as a defining feature of microbiota (Ley et al., 2006; Mariat et al., 2009), even though both bacterial phyla contain a wide range of organisms with distinct ecological roles (Qin et al., 2012). Microbial community ecology has the potential to identify key interactions between microorganisms, with a wide range of important applications in health and the environment (Bakker et al., 2012; Costello et al., 2012; Preidis and Versalovic, 2009). The below text will detail the approaches use to investigate microbial interactions.

2.2.2.1. Omic-approaches use to study microbial interaction

In the biological sciences, the suffix **-omics** is used to refer to the study of large sets of biological molecules (Smith et al., 2005). The idea is the field of molecular biology needed to move from studying isolated biological molecules towards a broad analysis of large sets of biological molecules (Sachidanandam et al., 2001). The OMICS field ranges from genomics (focused on the genome) to proteomics (focused on large sets of proteins, the proteome) and metabolomics (focused on large sets of small molecules, the metabolome). The field of genomics is divided into genotyping (focused on the genome sequence), transcriptomics (focused on genomic expression) and epigenomics (focused on epigenetic regulation of genome expression) (Vlaanderen et al., 2010). Currently, five different OMICS fields are well established: genotyping, gene expression profiling, epigenomics, proteomics, and metabolomics. In parallel to omics approaches, the systems biology community has been developing tools to simulate the complete metabolic network activity of individual microbes and natural or engineered microbial communities (Klitgord and Segrè, 2011; Zomorrodi and Segrè, 2016).

A. GENOMICS

These tools are focused on a predictive, quantitative understanding of metabolite-mediated interactions between species using extensions of flux balance analysis (FBA) (Orth et al., 2010). Metagenomics can be defined as the study of the whole genetic material of the microbial community existing in certain eco-environments (Sleator et al., 2008). The ultimate goal of metagenomics is to acquire a global view of the composition and function of the microbial community (Guazzaroni et al., 2009). Metagenomics is culture-independent studies of the collective set of genomes of mixed microbial communities and applies to exploitations of all microbial genomes in community that reside in environmental niches, in plants, or in animal hosts (**Figure 2-3**). One issue is that the science of metagenomics, in contrast to individual microbial

or animal genomes, is ultracomplex and challenged by the existence of vast unknown or knowledge “deserts”. Due to extremely large microbial taxonomic in nature, only a restricted set of bacterial populations have been identified. As an example, the colonic microbiota is a vast ecosystem with approximately 800–1000 species per individual, but these estimates are in flux because the science of metagenomics and microbial pan-arrays is so new. Approximately 62% of the bacteria identified from the human intestine were previously unknown, and 80% of the bacteria identified by metagenomic sequencing were considered non-cultivatable (Eckburg et al., 2005).

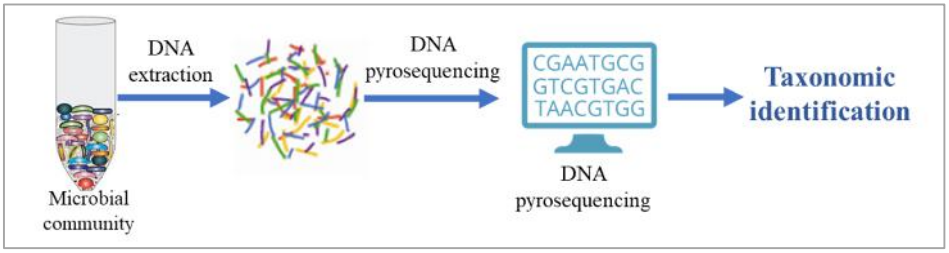

Figure 2-3: Summary of steps in metagenomic approach.

B. TRANSCRIPTOMICS

The transcriptome is the set of all RNA molecules, including mRNA, rRNA, tRNA, and other noncoding RNA, isolated from one cell or a population of cells (Manning et al., 2007). Gene expression profiling is the identification and characterization of the mixture of mRNA that is present in a specific sample. An important application of gene expression profiling is to associate differences in mRNA mixtures originating from different groups of individuals to phenotypic differences between the groups. In contrast to genotyping, gene expression profiling allows characterization of the level of gene expression. The transcriptome in contrast to the genome is highly variable over time, between cell types and will change in response to environmental changes. A gene expression profile provides a quantitative overview of the mRNA transcripts that were present in a sample at the time of collection. Therefore, gene expression

profiling can be used to determine which genes are differently expressed as a result of changes in environmental conditions.

C. PROTEOMICS

Another way to explore the microbial interaction is the meta-proteomic approach. Similar to genotyping and gene expression tool, it is only the first step toward a comprehensive understanding of composition, dynamics and function of consortia. However, sequencing will not allow to understand the protein activity and the dynamic change (Nelson, 2008). Post-genomic molecular approaches such as proteomics admit studying the ultimate functional products of genes/genomes. The collective study of all proteins in microbial communities is referred as '**meta-proteomics'**, to distinguish from the proteomics study of single species. Protein expression levels represent the balance between translation and degradation of proteins in during gene expression. It is therefore assumed that the abundance of a specific protein is related to its role in cell function. However, the proteomic analysis is complicated by the high dynamic protein expression. Generally, the function of cells can be described by the proteins that are present in the intra- and intercellular space and the abundance of these proteins (Sellers and Yates, 2003). Although all proteins are based on mRNA precursors, post-translational modifications (PTMs) and environmental interactions make it impossible to predict abundance of specific proteins based on gene expression analysis alone. Meta-proteomics allows the measurement of gene expression from the perspective of presence and abundance of translated proteins (Blackstock and Weir, 1999; Wilmes and Bond, 2004). The proteomics platform can be generally classified as gel-free or gel-based systems (Kan et al., 2006). The traditional approach is to analyse the protein sample with two-dimensional poly- acrylamide gel electrophoresis (2D-PAGE) at first and then further cut the spot for MS-based protein identification. The MS techniques that can be used for protein identification include both matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI). MALDI is often coupled with time-of-

flight (TOF) mass analyser, while ESI can be coupled with a variety of mass analysers. The pattern of peptide distribution will be searched against a database of candidate proteins for identification. Even though the method was successfully applied for protein identification in gel-based proteomics, the accuracy and reproducibility of the method is often inconsistent. Proteomics provides insights into the role proteins have in biological systems. A major challenge is the high variability in proteins and protein abundance in certain types of biological samples.

D. METABOLOMICS

Metabolic phenotypes are the by-products that result from the interaction between genetic, environmental, lifestyle and other factors (Holmes et al., 2008). Lipids or vitamins were counted as small molecules known as metabolite. Metabolomics is the study of metabolic profiles in collective biological samples. (Claudino et al., 2007). The metabolome analysis is highly variable due to time dependent and wide range of chemical structure. A crucial challenge of metabolomic is to obtain qualitative and quantitative information regarding the metabolites that occur under normal circumstances to be able to investigate perturbations in the complement of metabolites as a result of changes in environmental factors.

2.2.2.2. Molecular fingerprinting techniques

For microbial diversity analysis, molecular fingerprinting techniques are usually used to analyse the sequence of 16S rRNA from different microbial species, where both molecular fingerprints and phylogenetic affiliation of microbial species can be generated (Smalla, 2004). These techniques have been proven to be helpful in providing an overview of microbial diversity. Among the different mentioned genetic finger-printing techniques, DGGE is perhaps the most commonly used. Recently, DGGE was employed to explore microbial diversity in herbivore insects to study the potential mechanisms for biomass degradation. Another traditional molecular fingerprinting technique is random amplified polymorphic DNA (RAPD). The

analysis is based on amplification of genomic DNA using random primers. The discovery highlighted that symbiotic microbiota can be indigenous instead of exogenous from the food material (De Vries et al., 2001a, 2001b). The application of RAPD is also very limited due to low reproducibility of the technique. Single-strand conformation polymorphism (SSCP) is a technique that uses electrophoresis to separate single-strand DNA to differentiate the homologous sequences (Yandell, 1991).

2.2.2.3. Fluorescent in situ hybridization

In the last decade, fluorescence in situ hybridization (FISH) became the method of choice for the direct detection and identification of microorganisms (Amann and Ludwig, 2000; Moter and Göbel, 2000; Xufre et al., 2006) since it combines the direct visualization with the reliability of molecular methods (**Figure 2-4**). FISH technology based on 16S rRNA directed, fluorochrome-labelled oligonucleotide probes was usually employed and accompanied with confocal laser scanning microscopy (CLSM) allowing temporal detailed analysis of the three-dimensional (3D) spatial structure of this community. Once complex bacterial communities from environmental samples are analysed by FISH with rRNA-targeted probes, several technical problems might happen, and the detailed composition of the microbiota cannot be revealed. In addition, bacteria in less nutrient-rich environments have low ribosome content, which will affect the sensitivity of detection (Smalla, 2004). Beside FISH, DAPI (4,6-diamidino-2-phenylindole) and GFP (green fluorescent protein) have been used to visualize microbial communities (Cazemier et al., 1997a,b).

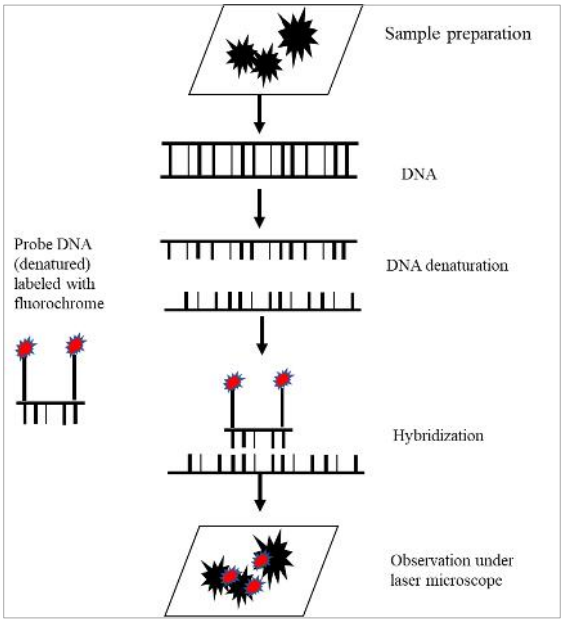

Figure 2-4: Flow chart of a typical Fluorescence in situ hybridization procedure

2.3. Biofilm reactors to promote microbial interactions

2.3.1. Biofilm formation

Historically, many studies have assumed that microbes lead solitary, asocial lives. On the contrary, bacteria predominantly live in dense biofilm populations interacting extensively with each other (Nadell et al., 2008). Indeed, most bacteria in nature do not live individually but endure co-ordinately, spatially organized, and metabolically integrated biofilm communities. Biofilms are ubiquitous and can develop on virtually every natural and human-made surface. In natural habitats, biofilms generally provide homeostasis in the face of fluctuating and harsh environmental conditions (Hall-Stoodley et al., 2004). Biofilms can comprise a single microbial species or multiple microbial species and can form on a range of biotic and abiotic surface. The development of a microbial biofilm have been shown as a dynamic process involving successive steps **Figure 2-5**. The first step is the attachment of the cells to the selected abiotic or biotic surface. Generally, bacterial cell or conidia spore attach to a

substratum that can promote the adherence to the surface. Initially, the attachment is mediated with very weak reversible van der Waals interaction between the cell surface and the substratum. Bacteria usually adhere to a conditioning film typically composed of organic molecules (e.g. nutrients, salivary proteins, large macromolecules) that can promote the adherence of bacteria to the surface (Donlan, 2002).

The second step corresponds to the development of micro colonizer promoted by the growth and division of the first attached cells. Then, the biofilm is formed in the third stage. In the early stages, these microcolonies can also disperse and/or move as a unit across a surface, further emphasizing the dynamic and somewhat transient early interactions of bacteria with a surface and each other (O'Toole et al., 2000). Biofilm become matures, and cells form multi-layered clusters. In stage 4, three-dimensional growth and further maturation of biofilm. After attachment to a surface, bacteria undergo further adaptation to life in a biofilm. Two properties are often associated with surface-attached bacteria, one is increased synthesis of EPS and the development of antibiotic resistance. These features appear to create a protective environment. At the final stage, the biofilm become more matured and reaches a critical mass and disperses planktonic cell, those cells are ready to colonize other free surfaces. The biofilm developmental pathway that concerns detachment represents an important area of future research. One possible signal for detaching may be starvation of the cell, although this has not been investigated in detail (GA O'Toole, unpublished data).

Biofilm comprises two kind of cells, planktonic cells were named for the free suspended cells and sessile for the irreversible attached cells on the surface. During biofilm development, sessile cells acquire physiological characteristic differentiating from planktonic cells. These two phases are in balance because of cellular adhesion from planktonic to sessile phase, biofilm detaches to planktonic phase and cells desorption.

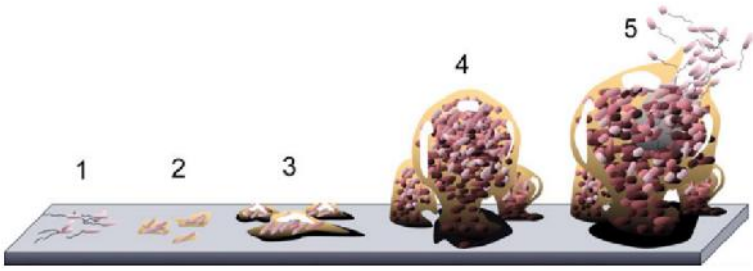

Figure 2-5: The five stages of biofilm development on biomaterial surface.

Stage 1: planktonic (free floating) bacteria adhere to the substratum surface. Stage 2: cells aggregate, form micro colonies and excrete extracellular polymeric substances (eps). Stage 3: a biofilm is formed. It matures, and cells form multi-layered clusters. Stage 4: three-dimensional growth and further maturation of the biofilm. Stage 5: the biofilm reaches a critical mass and disperses planktonic bacteria, ready to colonize other surfaces. (Monroe, 2007).

Microorganism account less than 10 % of the total dried mass of most biofilm, meanwhile the matrix can rise to more than 90%. The matrix is the extracellular material, mostly produced by the microbes themselves. That matrix has been known as known as extracellular polymeric substances (EPS) **Figure 2-6**. Most bacteria can produce polysaccharides, either as wall polysaccharides (capsules) or as extracellular excretions into the surrounding environment (EPS). It is most likely that EPS plays various important roles in the structure organization of biofilm and function of different biofilm matrix communities (Davey and O'toole, 2000). The EPS matrix also has the potential to physically prevent access of certain antimicrobial agents into the biofilm by acting as an ion exchanger, thereby restricting diffusion of compounds from the surrounding environment into the biofilm (Gilbert et al., 1997).

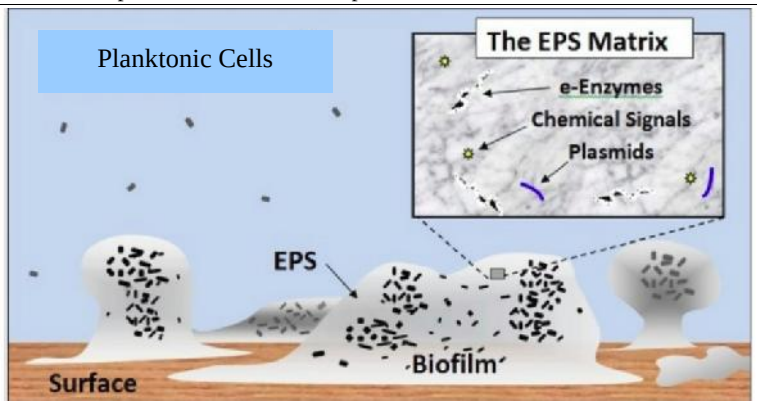

Figure 2-6: Biofilm composed of attached microbial cells encased within a matrix of extracellular polymeric secretion (eps) which surround and protect cells. EPS matrix is typically consisting of polysaccharides, proteins, lipids and extracellular DNA.

2.3.2. Approach to studying biofilms

To identify and characterize the properties that are necessary for biofilm development, a simple genetic screen has been used by several studies (Genevaux et al., 1996; Heilmann et al., 1996a, 1996b; Mack et al., 1994; O'Toole and Kolter, 1998). Biofilms are visualized with a variety of dyes (such as crystal violet and safarinin). The simplicity of the assay has meant that high-throughput screens of many thousands of randomly generated mutants have been carried out with relative ease. In addition, rapid progress has been developed in identifying certain bacterial structural components and sensory systems that are necessary for the initiation of biofilm formation. The method of extracting morphological from images of biofilm in order to quantify the characteristic of the inherent heterogeneity has evaluated. This is the early step towards quantifying the relationship between heterogeneity and the underlying processes, such as mass-transport dynamics, substrate concentrations, and species variations (Yang et al., 2000). Biofilm models are also commonly used as simulation tools in engineering applications and as research tools to identify and fill gaps in understanding of biofilm processes (NOGUERA et al., 1999).

2.3.3. Attachment of biofilm on the substratum

The solid-liquid interface between a surface and an aqueous medium (e.g., water, blood) provides an ideal environment for the attachment and growth of microorganisms. Several factor to be considered to get a clear picture of attachment including the effects of the substratum, conditioning films forming on the substratum, hydrodynamics of the aqueous medium, characteristics of the medium, and various properties of the cell surface. The solid surface may have several characteristics that are important in the attachment process. The extent of microbial colonization appears to increase once the surface roughness increases. This is because shear stress declined, and surface area is higher on rougher surfaces. The physicochemical properties of the surface may also exert a strong influence on the rate and extent of attachment. Most investigators have found that microorganisms attach more easily and rapidly to hydrophobic, nonpolar surfaces than hydrophilic materials (Bendinger et al., 1993; Fletcher and Loeb, 1979). A material surface exposed in an aqueous medium will inevitably and almost immediately become conditioned or coated by polymers from that medium, and the resulting chemical modification will affect the rate and extent of microbial attachment (Loeb and Neihof, 1975). The surface energy of the suspending medium may also affect hydrodynamic interactions of microbial cells with surfaces by altering the substratum characteristics. The flow velocity immediately adjacent to the substratum/liquid interface is negligible. For flow regimes characterized as laminar or turbulent, the hydrodynamic boundary layer may substantially affect cell-substratum interactions. Cells behave as particles in a liquid, and the rate of settling and association with a submerged surface will depend greatly on the velocity characteristic of the liquid. The characteristics of the aqueous medium, such as pH, nutrient, ionic strength, and temperature, also play a role in the rate of microbial attachment to a substratum. Several studies revealed the effect on bacterial attachment and biofilm formation in the different aqueous systems (Donlan et al.,

1994; Fera et al., 1989). Fletcher (1988) showed that an increase in the concentration of several cations affected the attachment of *Pseudomonas fluorescens* to glass surfaces, presumably by reducing the repulsive forces between negative charge of bacteria and the glass surface.

Cell surface hydrophobicity, presence of fimbriae and flagella, and production of EPS all influence the rate and extent of attachment of microbial cells. The hydrophobicity of the cell surface is important in adhesion because hydrophobic interactions tend to increase with an increasing nonpolar nature of one or both surfaces involved (i.e., the microbial cell surface and the substratum surface). Most bacteria are negatively charged but still contain hydrophobic surface properties (Rosenberg and Kjelleberg, 1986). Other cell surface properties may also facilitate attachment. Several studies have shown that treatment of adsorbed cells with proteolytic enzymes caused a marked release of attached bacteria, providing evidence for the role of proteins in attachment (Danielsson et al., 1977; Levanony and Bashan, 1991). Considering these findings, cell surface structures such as fimbriae, other proteins, LPS, EPS, and flagella all clearly play an important role in the attachment process. Cell surface polymers with nonpolar sites such as fimbriae, other proteins, and components of certain Gram-positive bacteria (mycolic acids) appear to dominate attachment to hydrophobic substrata, while EPS and lipopolysaccharides are more important in attachment to hydrophilic materials. The attachment of microorganisms to surfaces is a very intricated mechanism, with many factors affecting the outcome. In general, attachment will occur most readily on surfaces that are rougher, more hydrophobic, and coated by surface “conditioning” films. A rise in flow velocity, water temperature, or nutrient concentration may also associate to increased attachment, if these factors do not exceed critical condition. Physicochemical properties of the cell surface, specifically the presence of fimbriae, flagella, and surface-associated polysaccharides or proteins, are also crucial and may probably provide a competitive advantage for one organism where a mixed community is involved.

2.3.4. Devices for biofilm cultivation

A thin layer of settled biomass composed of several microbial species which are subject to interactions, such as symbiosis or competition for space or for common substrates, is called a multispecies biofilm. Its microbial composition (i.e., both the relative abundance and spatial distribution of the species) is mainly determined by three processes that take place within the film: (i) microbial conversion of substrates; (ii) volume expansion of biomass; and (iii) transport of substrates by molecular diffusion. Biofilms often consist of multiple different species of bacteria residing near one another. Interspecies interactions in multispecies biofilm may result in emergent functions and capabilities, emphasizing the relevance of further studying these complex communities. Regarding to microbial distribution, in fact, many researches focusing on performance of multispecies biofilms is either based on indirect experimental evidence or on a priori assumptions. In recent years, abundant studies examining multispecies biofilms has increased to highlight the need for appropriate experiments for investigating microbial interactions in the complex communities, these studies are relevant to multiple areas, including medicine, industry, and ecology.

Several type of biofilm cultivation devices can used and proposed such as Microtiter plates, Robbin device, Flow chamber reactor, Drip flow biofilm reactor, Rotary biofilm reactors, Biofilm microfluidic devices, **Figure 2-7** (Azeredo et al., 2017). Indeed, those mentioned devices allow to observe biofilm development, the interactions of multispecies biofilm with several environmental factors influencing the biofilm formation.

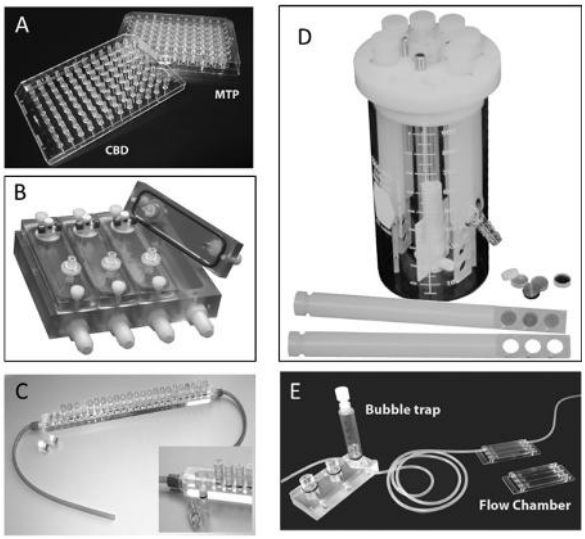

Figure 2-7: Illustration of five cultivation devices.

A: Microtiter plates and Calgary biofilm device, B: Drip flow biofilm reactor, C: The modified Robbins device, D: Rotary biofilm reactors, E: Flow chamber system (Azeredo et al., 2017).

Microtiter plates: The most commonly used devices for biofilm formation are microtiter plates. Indeed, this device is potential and useful because it contains enormous tube wells in one microtiter plate which is good replicated experiment. At the different time of cultivation, several well has been removed the planktonic phase and determined the settled biomass. In the microtiter plate assay, the biofilm biomass is assessed by measuring all attached biomass. However, biomass sedimented to the bottom of the tube wells may embedded with extracellular polymeric substances (EPS). Thus, the result of total settled biofilm does not represent only the biomass (Azeredo et al., 2017; Djordjevic et al., 2002). To overcome this problem, another kind of microplates have been designed, Calgary biofilm device (Ceri et al., 1999). This device has principally the same frame as microtiter plates with inserted pegs in wells. The biofilm formed on the pegs does not result from cell sedimentation but only from sessile development. The biofilm typically forms on pegs which are inserted in

the meddle of medium. The biofilm is removed afterward from the pegs by using sonication for further quantification (Edmonds et al., 2009; Müller et al., 2011). Generally, only a fraction of the sessile community can be suspended by sonication, typically between 5% and 90% of the. Second, the physiological properties of the detached population may not reflect the physiology of sessile cells, as different populations could exhibit different adhesive and detachment properties on the material (Grand et al., 2011). In both microtiter-based assays, the wells or pegs can further be coated with different molecules to investigate or promote biofilm formation on different biotic and abiotic supports.

***Drip flow biofilm reactor*:** The drip flow biofilm reactor was developed by Darla Goeres and his research team from the Center for Biofilm Engineering, Montana State University (Goeres et al., 2009). This device principally composed with several chambers which are available to test with different support materials (coupon). The drip flow reactor (DFR) is a useful tool to support growing biofilm under low shear force at an air-liquid interface and continuous culture mode. Two steps are necessary for the study of biofilm development: static phase and a continuous phase. In static phase, each chamber containing coupon and inoculated with microbial growth medium or cell suspension tilts 10^{o} from horizontal for an appropriate time (Agostinho et al., 2011; Buckingham-Meyer et al., 2007; Stewart et al., 2001). Then, spent media is entirely removed, theoretically leaving the surface with only the adhered cells or spores. Then, the continuous phase starts by addition of fresh nutritive medium into the inert support with a low flow rate. The first phase is used for the surface attachment of the cells and the second step allows the biofilm formation on coupons without immersing the cells. The drip flow biofilm reactor has several advantages such as small space needed, easy operation, simultaneous use of different surface materials and possibility to analyse samples noninvasively. Therefore, this reactor has been extensively used for different assays, e.g. to assess the effect of disinfection strategies, to enhance the biofilm formation and nanocomposites on biofilm control under low

shear stress at an air-liquid interface (Buckingham-Meyer et al., 2007; Sawant et al., 2013; Stewart and Costerton, 2001) to mimic indwelling medical devices and evaluate antimicrobial properties (Ammons et al., 2011; Carlson et al., 2008). Recently, there was an interesting study focus on microbial interaction within the biofilm stains by using drip flow biofilm device to visualize the organization of each species (Liu et al., 2016). The Limitations of this device are biofilm heterogeneity on the coupons associated with the device hydrodynamic and the low shear stress and low similarity with industrial conditions.

Robbins device: The Robbins device is based on the design of Jim Robbins and Bill McCoy, and later patented in a revised version by the Shell Oil Company. It consists of a pipe with numerous holes where coupons are mounted on the end of screws placed into liquid medium flow (Azeredo et al., 2017; McCoy et al., 1981). This device is generally used to monitor biofilm formation under different fluid velocities in a simulated drinking water facility (McCoy et al., 1981; Nickel et al., 1985). The modified Robbins device consisted of a square channel pipe with equally-spaced sampling ports attached to sampling plugs aligned with the inner surface, without disturbing the flow characteristics **Figure 2-7-D,** a considerable advance over the original device. Several hydrodynamic conditions can be studied with this device such as the laminar and turbulent flow (Linton et al., 1999). The applications of the modified Robbins device are large, from biomedical to industrial scenarios. However, because the device is not designed to allow direct observation of the biofilm development, coupons must be removed for examination. The Robbins device has been used extensively to investigate the immune response to biofilm-grown bacteria and antibiotic resistance. This technique has provided much useful information on the physiology and metabolism of biofilm bacteria, regulation of various bacterial genes and their products, and resistance to antimicrobial compounds and their interactions with various components of the immune system (Kharazmi et al., 1999).

Rotary biofilm reactors: There are three different kinds of rotary biofilm reactors including the rotary annular reactor, the rotary disk reactor and the concentric cylinder reactor. Annular reactor is composed of two cylinders, a static outer one and a moving/rotating inner other. The adjustable speed motor control the rotation frequency of the internal cylinder; thus, a well-mixed liquid phase, turbulent flow with constant shear force fields may be obtained (Lawrence et al., 2000). These reactors use retrievable coupon surfaces where the biofilms develop and enable chemical and biochemical analyses as well as microscopy observation of the biofilms. The rotary disk reactor consists a disk which is designed to support several coupons. The disk rotation creates a liquid surface shear across the coupons and as they are placed at the same radial distance; thus, they will experience a similar shear stress field. This reactor has been used to study biofilm resistance, to develop biofilm control or to evaluate interspecies interactions in multispecies biofilms (Coenye and Nelis, 2010). This configuration is principally used for the study of the impacts of the shear stress on the biofilm distribution and development (Willcock et al., 2000). The advantages of these devices are that the biofilms are formed in relatively controlled shear stress and in the case of the rotary annular reactor and the rotary disk reactor different surfaces of coupons can be tested simultaneously. Thus, coupons can be made from different materials such as PVC, steel, plastics… The disadvantage of these reactors is that the number of individual strains that can be analyzed simultaneously is low as only one per experiment in the rotary annular reactor and rotary disk reactor and up to four can be used in the concentric cylinder reactor. Another weakness it that due to the semi-open design, contamination problems are common, and these may be difficult to sort out. Thus, high-throughput analysis of large numbers of strains is not possible with these systems.

Flow chamber system: A parallel-plate fluid flow chamber system is a benchtop (in vitro) model that simulates fluid shear stresses on various cell types exposed to dynamic fluid flow in their natural, physiological environment. The biofilm is

encapsulated in a reactor (flow) chamber with an inspection glass or plastic window onto which the biofilm can develop. Then, the microscope lens can record images from the substratum side of the biofilm. The liquid flows from one chamber, across the coupon and is collected in the other chamber. Fresh medium is supplied continuously and typically recycled several times before a fraction is purged. This system is quite flexible but allows only single experiments to be carried out at one time and requires a large volume of medium. The advantage of this system is allowing direct access to the biofilm for manipulation or sampling. The flow chamber is sealed with a microscope cover glass. Media flows through the channels while microscopic examination can be performed on-line. Depending on the geometry of the flow chamber and the flow rate, the flow may be laminar or turbulent which can influence the distribution of nutrients and dismissal of waste products, and ultimately biofilm structure (Skolimowski et al., 2010; Stoodley et al., 1998).

2.3.5. Interspecies bacterial interactions in biofilms reactor

Bacteria and unicellular eukaryotes, such as yeasts and filamentous fungi, are found together in a innumerable of environments and exhibit both synergistic and antagonistic interactions (GARBAYE, 1994; Inbar and Chet, 1995). Interspecies interactions appear to have a preponderant role in natural ecosystems. Indeed, the composition of microbial communities, which populate most environments, is largely shaped by interspecies competition or cooperation (Guillonneau et al., 2018). The utilization of microbial community to obtain desirable features has increase over the past few decades (Berlanga and Guerrero, 2016; Brenner et al., 2007; Dang and Lovell, 2016; Großkopf and Soyer, 2014). In the field of biotechnology advancement, the key interaction within microbial consortia have been increasing which has led to improve the application and design for biotechnological process in natural and engineered systems. Interspecies interactions may also result in di erent microbial functions and abilities (Røder et al., 2016). Very limited information except for the

human oral cavities, where multispecies studies are the most abundant (Bloch et al., 2017; Filoche et al., 2004; Kolenbrander et al., 2010; Kuboniwa et al., 2006). Regarding the utilization of interkingdom co-cultures, phylogenetically distant species can be gathered to gain novel functionality and higher production efficiency ((Lindemann et al., 2016; Muñoz et al., 2006). Several disciplines and fields can benefit from these microbial consortia, including environmental engineering, biosynthesis of fuel and production of commodity chemicals (Bernstein and Carlson, 2012; Minty et al., 2013). Bacterial interactions begin to influence a biofilm during the initial stages of biofilm development. The formation of a biofilm begins with the adsorption of molecules to a surface followed by bacterial adhesion and colonization. Interspecies interaction, including co-metabolism, quorum sensing and production of antimicrobial compounds have been shown to play important roles in regulating microbial activities in vitro (Hojo et al., 2009) and have important functions in shaping the spatial structure of biofilms. In accordance, Christensen et al., (2002) observed this phenomenon by performing a study showing the shift from weak cooperation to exploitation of two species co-cultured in biofilm. This leads to a layered structure with patchy patterning of species as the biomass increased, with the benefitting species overgrowing the one being benefitted from. Hansen et al., (2007) also found a similar pattern formed between two species, as a result of a recent mutation in the overgrowing strain. This novel exploitative interaction could, however, be transient, since it generates a selective pressure on the producing strain to avoid being exploited. Competitive metabolic interactions among species often play critical roles in shaping the structure and function of multispecies communities. Scheffer and van Nes, (2006) demonstrated that self-organized organized segregation of species in communities is a direct effect of competitive interactions, using a model based on classical competition theory. Kim et al., (2008) and Momeni et al., (2014) indicated that biofilms with competitive pairs frequently show a spatial structure with interspecific segregation (equal-fitness competition) or one species dominant (unequal-fitness

competition). Metabolic interactions, leading to cooperation, exploitation, or competition, are ubiquitous in multispecies biofilm and play important roles in maintaining the diversity and stability of microbial communities (Embree et al., 2015; Møller et al., 1998; Zelezniak et al., 2015). The fitness effects of interspecific interactions are believed to be a major driving force of spatial organization of microbes in multispecies biofilms. Simulations derived from an individual-based fitness model showed that deviations caused by indirect interactions can obscure direct interactions and lead to misinterpretations (Momeni et al., 2013). Additionally, interspecific interactions between competitive pairs could shift to indirect cooperation for coexistence in response to the surrounding species or resources (Wootton, 2002). Additionally, the support can be considered as an efficient way for bioprocess intensification by promoting the exchanges between the biofilm, gas and liquid phases due to reduced energy consumption. In the field of biotechnological applications, single- and multi-species culture have a rise of interest in field of research in order to investigate new technology as well as interested metabolites (Liu et al., 2016; Ponomarova and Patil, 2015, Qureshi et al., 2005,)

3

RESULTS AND DISCUSSION

Chapter 3.1. Impact of microbial composition of Cambodian traditional dried starters (dombea) on flavour compounds of rice wine: combining amplicon sequencing with HP-SPME-GCMS.

This first part showed the impact of microbial composition of Cambodian traditional dried starter (*Dombea*) on flavour compounds of rice wine. Both bacterial and fungal diversity of five ferment starters were analysed using Pyrosequencing. Then, flavour compounds of final rice wine were analysed by SPME-GCMS. Correlation of the presence of species and flavour produced were investigated and visualised by SPSS and Cytoscape.

Chapter 3.2. Exploitation of microbial interactions in bioprocesses through the use of synthetic communities and biofilm for the alcoholic beverage.

This second part presented the impact of combination of selected strains on flavour compound prior to replace the natural community (*Dombea*). The flavour compound produced from synthetic and natural community were investigated and compared. The result from Chapter 3.2 has shown the importance of biofilm adhesion. Thus, to better understand and control the microbial community, the performances of synthetic community were studied in the packing system.

Chapter 3.3. Modulation of fungal biofilm physiology and secondary metabolites production based on surface physico-chemistry.

This last part detailed the effect of mode culture on the biofilm formation of *Trichoderma* spp. This result contributed the understanding on the performance of filamentous fungi; especially the adhesion, prior to easily scale up the bioprocess with interkingdom (LAB, Mould, Yeast) for rice wine production. In this part, the different culture system including shake flask and dripflow were discussed.

<u>Chapter 3.1:</u> Impact of Microbial Composition of Cambodian Traditional Dried Starters (*Dombea*) on Flavour Compounds of Rice Wine: Combining Amplicon Sequencing With HP-SPME-GCMS

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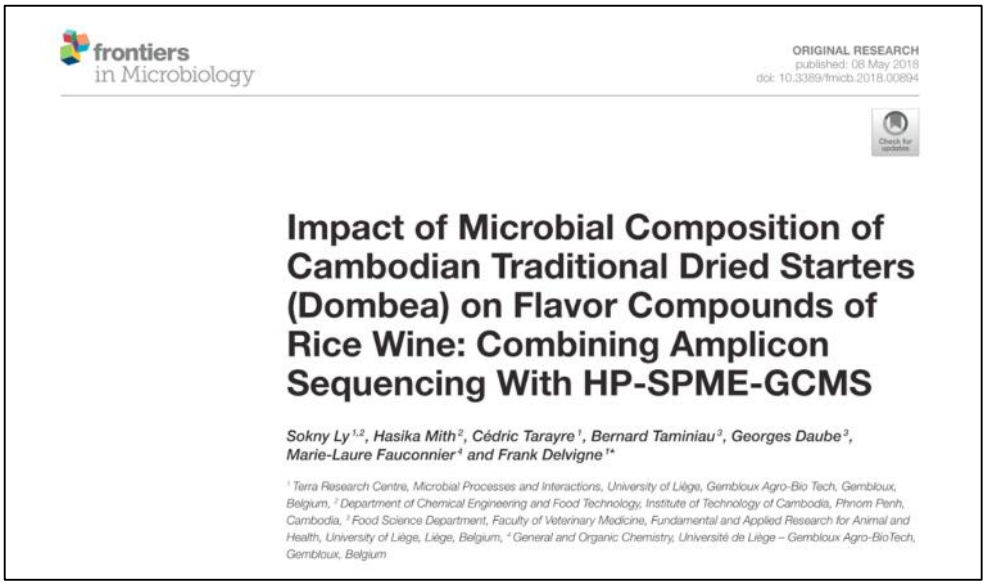

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Impact of Microbial Composition of Cambodian Traditional Dried Starters (Dombea) on Flavor Compounds of Rice Wine: Combining Amplicon Sequencing With HP-SPME-GCMS

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ABSTRACT

Dombae is a traditional ferment starter which has been used for starchy based wine production in Cambodia. However, the production technology of rice wine in Cambodia is not optimized. The current study aimed to investigate the microbiota associated in five ferment starters and the effect of a traditional fermentation process using a metagenomics sequencing analysis and HS-SPME-GCMS for the characterization of the aromatic profiles at the end of fermentation. Most of bacteria identified in this study were lactic acid bacteria including *Weissella cibaria, Pediococcus* spp, *Lactobacillus fermentum* and *Lactobacillus plantarum. Saccharomyces cerevisiae* and *Saccharomycopsis fibuligera* were found to be abundant yeasts while the only amylolytic filamentous fungus was *Rhizopus oryzae*. A total of 25 aromatic compounds were detected and identified as esters, alcohols, acids, ketones and aldehydes. The alcohol group was dominant in each rice wine. The changes and the potential functions of microbial communities in the starters and the modifications caused by the fermentation process were discussed. The presence of *Weissella, Pediococcus* and *Lactobacillus* genus had strong correlation with most flavour compounds.

KEYWORDS: rice wine fermentation, microbial communities, dried starter, SPME-GCMS, amplicon sequencing

INTRODUCTION

The fermentation process allows to preserve and to enhance the nutritional value of food resources. All over the world, human societies without exception found the way of making fermented beverages from sugar sources available in their local habitats. Similarly, Cambodian people apply the traditional fermentation beverage process to many raw materials such as rice, cassava and other starchy resources. Rice-based fermented beverage have been known or named as rice wine in most Asian countries

such as following: in India (Jeyaram et al., 2008b), in Thailand (Chuenchomrat et al., 2008), in China (Wang et al., 2014), in Korea (Kim et al., 2011), in Vietnam (Dung et al., 2005). Beside non-sticky rice, red rice is also used to produce wine (red rice wine), which is particularly desired for its brown-red colour and special fruity aromas. Its uncommon characteristics in comparison to the colourless wine from white rice make it much more attractive. Furthermore, red rice contains polyphenols and anthocyanins, which have been reported to be highly effective cholesterol treatment in the human body and to inhibit the growth of tumour cells (Sompong et al., 2011). Microbial ferment starter, under the form of dried powders or hard ball made from starchy cereals, are used to induce alcoholic fermentation. These starters' preparations have different names such as *Loogpang* in Thailand, *Bubod* in Philippines, *Marcha* in India and Nepal (Sha et al., 2017), *Ragi* in Indonesia, Chinese yeast or *Chiuchu* in Taiwan (Ellis, 1985), *Nuruk* in Korea (Park et al., 2014) and *Medombae* or *Dombae* in Cambodia (Chim et al., 2015a). Both starter preparation and rice wine fermentation were first made in uncontrolled conditions and with different methods, depending on the wine maker.

The principle of rice wine production consists of saccharification of steamed starchy resource by fungi under solid state fermentation and by yeasts under submerged alcoholic fermentation (Blandino et al., 2003; Dung et al., 2007; Sujaya et al., 2004). These traditional processes in Cambodia lack research and optimization in the field of food technology. This optimization requires the food safety, the control of nutritional value, the improvement of production methods, the sustainable quality and the reduction of production costs. Rice wine producers regularly met the problems of a low yield of rice wine and the inconsistency of quality in terms of taste and flavour. The nature of microbial communities in Cambodian traditional starters, their interactions and their contributions to the synthesis of aromas during fermentation are still widely unknown. Several studies were previously focused on the microbial diversity in ferment starters (Chao et al., 2013; Ercolini, 2004; Jeyaram et al., 2008b;

Luangkhlaypho et al., 2014; Lv et al., 2012a; X. C. Lv et al., 2015; Sha et al., 2017; Thanh et al., 2008; Wang et al., 2014; Xie et al., 2013). A very few studies were investigated on the ferment starters and the fermentation process in Cambodia. Therefore, the objective of this study was not only to investigate the composition of microbial communities in dried starters but also their evolution after the fermentation process. Furthermore, the aromatic profiles of each rice wine were analysed to understand the different flavours of rice wines depending on the type of starter.

MATERIALS AND METHODS

Sample collection

The Cambodian traditional starters were produced through different methods. The starters were collected from five different regions in Cambodia and labelled as DBB, DCK, DOB, DOS and DPK. The red rice used in this study was purchased from only one region and was freshly harvested. The samples were stored in the laboratory at 4°C or -20°C for further analyses.

Processing of red rice wine fermentation

The laboratory scale processing of red rice wine production was adapted from the traditional process by local rice wine producers. Briefly, 100 g of red rice were soaked in distilled water for three hours. A volume of distilled water was then added and steamed in an autoclave at 120°C for 20 min. The gelatinized rice paste was cooled to room temperature, then inoculated and mixed with 2% of traditional dried starter before being incubated at 30°C. After a solid-state aerobic fungal fermentation of three days, another volume of sterilized water was added to boost the alcoholic fermentation for other seven days more in the same flask. The fermented rice mashes were homogenized and the sampling was made every 24 hours.

Sugar and ethanol analysis by HPLC

The concentrations of maltotriose, maltose, glucose and ethanol were determined using RID-HPLC (Agilent 1100 series, Agilent Technologies). A volume of 5 µL was injected, in duplicate, through a Rezex ROA-Organic Acid column (300 x 7.8 mm) with 5mM H_2SO_4 as mobile phase at a flow rate of 0.6 mL/min at 60ºC.

Aromatic compounds analysis by HS-SPME-GC-MS

Rice wine mash was collected to analyse the aromatic compounds immediately after 10 days of fermentation. A 50 µm DVB/CAR/PDMS (Supleco, Bellofonte, PA, USA) was used as the extract fibre coating to perform the Headspace Solid-Phase Micro-extraction. The fibre was conditioned according to the manufacturer's instructions. A volume of 5 mL of rice wine sample with 30% NaCl and 1 µL Octan-2-ol (80.2 mg/L prepared in absolute ethanol) as internal standard were added into a 20 mL screw cap glass vial containing a magnetic stirring bar. The final concentration of octan-2-ol was 1.6 mg/L. The fibre was exposed to the sample containing vial for 30 min at 60 ºC, after 30 min of equilibration. For all experiments, the desorption was done in the splitless mode using helium at a flow rate of 50 mL/min. The identification of the extracted analytes was performed in an Agilent 6890 GC with a VF-WAXms capillary column (30mm., 0.25mm I.D., 0.25 mm film thickness, Agilent Technologies). The carrier gas was helium at a flow rate of 1.9 mL/min. The injector temperature was at 250 ºC. The mass detector operated in the electron impact mode at 70 eV in a range from 35 amu to 400 amu, and the ion source temperature was set at 230 ºC. The oven temperature was held at 35 ºC for 2min, raised at 5 ºC/min to 155 ºC, then raised to 250 ºC at a rate of 20 ºC/ min, and held at 250 ºC for 10 min. The aromatic components were identified by comparison of their Retention Indices with data reported in the literature and their mass spectra to the NIST 05 data base (matching quality higher than 90%). The Retention Indices (RI) of unknown compound were calculated by the retention time of a series of alkanes (C5-C35). A semi-quantification of the volatile

compounds was performed using octan-2-ol as the internal standard. The quantification of each compound was performed if the peak represented more than 1% of the total area. The results were reported in the mean value of three biological replication of rice wine mash.

16S and 28S rDNA pyrosequencing

Total DNA was extracted from the stool samples with the PSP Spin Stool DNA Plus Kit 00310 (Invitek), following the manufacturer's recommendations. The DNA was eluted into DNase/RNase-free water and its concentration and purity were evaluated by absorbance measurement using the NanoDrop ND-1000 spectrophotometer (NanoDrop ND-1000, Isogen). PCR-amplification of the V1-V3 region of the 16S rDNA was performed. Primers targeting the 16S rRNA gene fragments E9-29, 5′-GAGAGTTTGATCATGGCTCAG-3′, and E514-530, 5′-ACCGCGGCTGCTGGCAC-3′ (Baker et al., 2003) were used for their theoretical ability to generate lowest possible amplification capability bias among the various bacteria. The oligonucleotide design included 454 Life Sciences' A or B sequencing titanium adapters (Roche Diagnostics) and multiplex identifiers (MIDs) fused to the 5′ end of each primer. PCR was performed in the following condition: the amplification mix contained 5 U FastStartHigh Fidelity DNA polymerase (Roche Diagnostics, Vilvoorde, Belgium), 1× enzyme reaction buffer, 200 μM dNTPs (Eurogentec, Liège, Belgium), each primer at 0.2 μM, and 100 ng genomic DNA in a final volume of 100 μL. Thermocycling conditions were denaturation at 94 °C for 15 min followed by 25 cycles of 94 °C for 40 s, 56 °C for 40 s, 72 °C for 1 min, and a final 7 min elongation step at 72 °C. The amplification was carried out on a Mastercycler ep Gradient thermocycler (Eppendorf, Ham- burg, Germany). The PCR products were electrophoresed through a 1 % agarose gel and the DNA fragments were plugged out and purified with the SV PCR Purification Kit (Promega Benelux). The quality and quantity of the products was assessed with a Picogreen dsDNA quantification assay.

All amplicons was sequenced with the Roche GS-Junior Genome Sequencer (Roche, Vilvoorde, Belgium). The same procedure was applied for fungi, except that a 500-pb fragment of the 28S rRNA gene was amplified and sequenced with the following primers: NL-1, 5′-GCATATCAATAAGCGGAGGAAAAG-3′, and NL-4, 5′-GGTCCGTGTTTCAAGACGG-3′ (Kurtzman and Robnett, 1997). All libraries were run in the same titanium pyrosequencing reaction using Roche multiplex identifiers, and amplicons were sequenced using the Roche GS-Junior Genome Sequencer instrument (Roche).

Bioinformatics analysis of the pyrosequencing products

The 16S and 28S rDNA sequence reads were processed using the MOTHUR software package (Schloss et al., 2009). The quality of all the sequence reads was assessed by using the PyroNoise algorithm implemented in MOTHUR and the data were screened according to the following criteria: minimal length of 425 bp, an exact match to the barcode, and one mismatch allowed for the proximal primer. ChimeraSlayer was used to check the sequences for the presence of chimeric amplification (Haas et al., 2011). The resulting reads were compared with a reference dataset (derived from the SILVA database) of full-length rRNA sequences implemented in MOTHUR. The final reads were clustered into operational taxonomic units (OTU) with the nearest neighbour algorithm using MOTHUR with a 0.03 distance unit cut-off. When taxonomic identification was below the 80 % threshold, the taxonomic level was labelled with the first defined level from higher level followed by the term "_unclassified". Population structure and community membership were assessed with MOTHUR using distance matrices based on the Jaccard index (a measure of community membership; which considers the number of shared OTUs but not their abundance) and the Yue and Clayton measure of dissimilarity (a measure of community structure which considers shared OTUs and their relative abundances) (Eshar and Weese, 2014). Richness estimation (Chao1 estimator) (Chao and Bunge, 2002), microbial

biodiversity (non-parametric (NP) Shannon diversity index) (Chao and Shen, 2003), and the population evenness (Shannon evenness) (Mudler et al., 2004) were calculated using MOTHUR. Chao 1 estimator was used to estimate the richness of the detected species (OTUs) in a sample (Delcenserie et al., 2014).

Statistical analysis

Five percentage from each strain presented in dried starter and corresponding flavour compounds were analysed their correlation with the significant level 95% by using SPSS v.23. Only the 24 bacterial and two yeast species were analysed due to limited value of others strains and those strains were not observed after fermentation. While the correlation with significant p-value were observed, those values were imported to Cytoscape Network software to visualize their interrelationship.

RESULTS

Bacterial communities in Cambodian traditional dried starters

The Cambodian traditional rice wine brewing process has been adapted at a lab scale. Five different Cambodian traditional starters were analysed as well as the microbial communities resulting from 10 days of fermentation. In this study, the genus and species labelling were addressed based on the V1-V3 region. The relative abundance of each genus and species was compared. As shown in **Table 1**, in terms of the overall species richness, the DPK dried starter showed the highest species abundance followed by DCK, DOB, DOS and DBB. Species richness represents the number of different species found in ecological community. The bacterial richness of DPK and DCK dropped from 166.33 and 156.41in the dried starter to 18.09 and 90.28; respectively, after the fermentation of 10 days. However, the bacterial richness of DBB, DOS and DOB increased slightly from 23.73, 49.17 and 93.58 to 27.51, 56.67, and 95.51, respectively. This showed that there were considerable changes in terms of bacterial species in the community after the fermentation stage for all type of

starters. The microbiota composition of each dried starter (before and after the fermentation) is presented at a genus level **(Figure 1)** and a species level **(Figure 2)**. Both genera and species of microbiota in the dried starters and after fermentation were complex but each dried starter had a specific microbial profile. The pyrosequencing analysis revealed that most bacterial genera were lactic acid bacteria including *Weissella* (ranging from 35 to 99% of the OTUs), *Lactobacillus* (ranging from 0 to 66% of the OTUs), *Pediococcus* (ranging from 0 to 39% of the OTUs), *Streptococcus* (ranging from 0 to 9% of the OTUs) and *Leuconostoc* (ranging from 0 to 5% of the OTUs).

Large changes in bacterial community have been observed between the dried starters and the microbial communities after fermentation **(Figure 2)**. During fermentation with the DBB starter, *Weissella cibaria*, which was prevalent in the starter, decreased slightly from 96.29% to 91.09% of the OTUs. However, *Pediococcus sp. MMZ60A* and *L. plantarum* considerably increased after the brewing process. Similarly, *L. plantarum* was found to be dominant in the DCK starter (57.93% of the OTUs) but not detected after the fermentation. Nevertheless, *L. fermentum* became prevalent (96.70% of the OTUs). In this starter, several species (*Streptococcus GV636515* (9%), *Leuconostoc garlicum* (5%) and *Acetobacteraceae liquefaciens* (4.7%) disappeared after the fermentation. In the DOB consortium, *W. cibaria* was prevalent. After the fermentation, *L. fermentum* and *L. plantarum* were dominant with respective OTU percentages of 65.29 and 25.24%. *Pediococcus sp. MMZ60A* was prevalent in rice wine after the fermentation stage performed by the consortia DOS and DPK. There was a remarkably impact on the bacterial community in DPK. *Pediococcus sp. MMZ60A* was present at 36.72% and got dominant (96.27% of the OTUs) after the fermentation. Moreover, *L. plantarum* was less detected in the dried starter DCK. However, it became the dominant bacterial species after the traditional fermentation (96.70%) while *L. fermentum* was detected (57.93% of the OTUs) in the dried starter and not detected after the fermentation. All these changes showed that the distribution

of bacteria varied and changed after the fermentation according to the traditional Cambodian process.

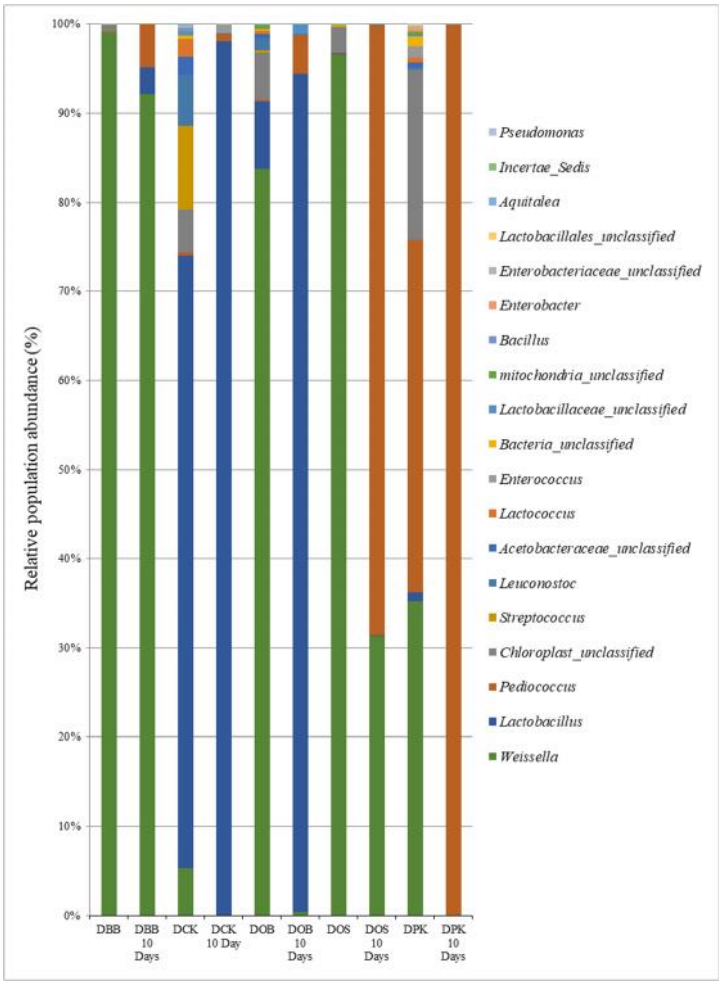

Figure 1: Bacterial composition (OTUs at the genus level based on 16S amplicon sequencing) of the five starters and the corresponding microbial communities after 10 days of fermentation (labelled “10 Days”).

Table 1: Bacterial diversity, bacterial richness and bacterial evenness of the five starters and the microbial communities after 10 days of fermentation.

Group	Bacterial diversity	Bacterial Richness	Bacterial Evenness
DBB	1.04	23.73	0.06
DBB 10 Days	1.18	27.51	0.06
DCK	2.67	156.41	0.03
DCK 10 Days	1.05	90.28	0.03
DOB	1.41	93.58	0.03
DOB 10 Days	1.98	95.51	0.03
DOS	1.20	49.17	0.04
DOS 10 Days	2.22	56.67	0.09
DPK	4.25	166.33	0.05
DPK 10 Days	1.06	18.09	0.09

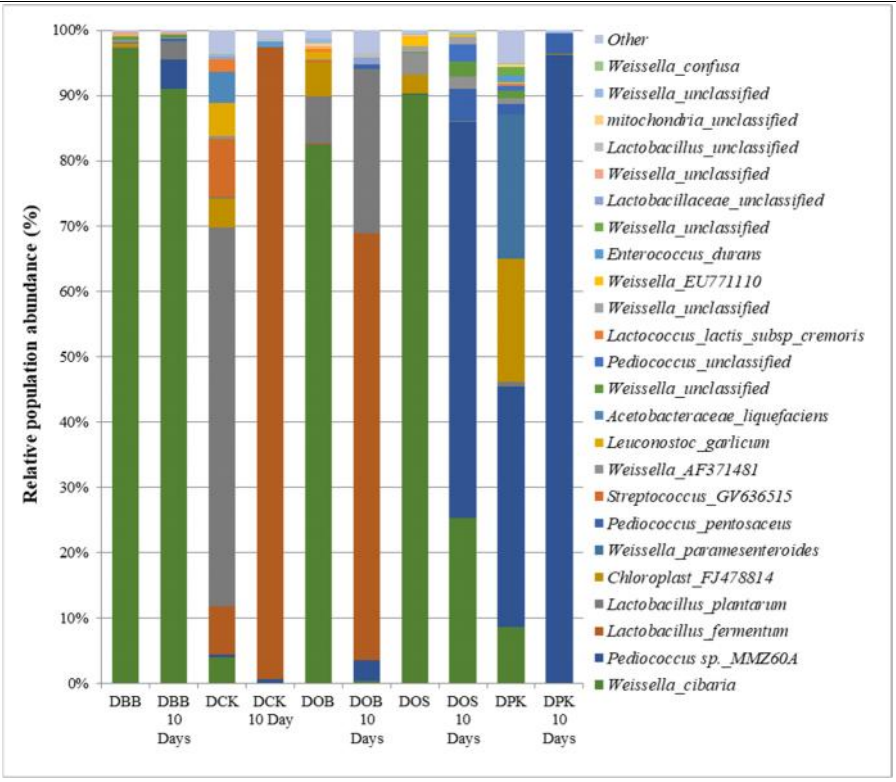

Figure 2: Bacterial composition (OTUs at the species level based on 16S amplicon sequencing) of the five starters and the corresponding microbial communities after 10 days of fermentation (labelled "10 Days").

Fungal community presented in Cambodian traditional dried starter

The fungal composition (in terms of OTUs) of the starters (before and after 10 days of brewing) is presented at the genus level (**Figure 3**) and the species level (**Figure 4**). Once more, strong modifications were observed. According to the results presented in **Table 2**, there were not many differences of fungal richness in the different types of dried starters. Therefore, it is believed that there were not many fungal species associated in the starter communities. After the fermentation stage, the rice wine obtained with the DCK and DOB starters led to the highest fungal richness (9.86 and 7.13, respectively). The fungal evenness, which refers to the uniformity of

the species inside the microbial community, was quite stable in each dried starter and also after the fermentation process.

Table 2: Fungal diversity, richness and evenness values in the five starters and in the fungal communities after 10 days of fermentation.

Group	Fungal Diversity	Fungal Richness	Fungal Evenness
DBB	1.081	2.682	0.425
DBB 10 Days	1.469	3.362	0.488
DCK	1.042	4.836	0.220
DCK 10 Days	1.003	9.866	0.152
DOB	1.001	3.000	0.335
DOB 10 Days	1.506	7.130	0.245
DOS	1.147	5.863	0.222
DOS 10 Days	1.030	4.279	0.278
DPK	1.000	1.228	0.886
DPK 10 Days	1.014	3.441	0.339

As shown in (**Figure 3**), the *Saccharomyces* genus was found ubiquitously as predominant (ranging from 55 to 87% of the OTUs) both in the dried starter and the fungal community after fermentation. It was observed that this genus increased in each brewing process. *Rhizopus* and *Saccharomycopsis* genus decreased after the fermentation. Many species were observed in the traditional dried starters in comparison to the communities after 10 days of fermentation (**Figure 4**). *Rhizopus* spp. was the only filamentous and amylolytic fungal genus found in all dried starters. *Rhizopus oryzae* was the predominant and represented more than 90% of OTUs in each dried starter.

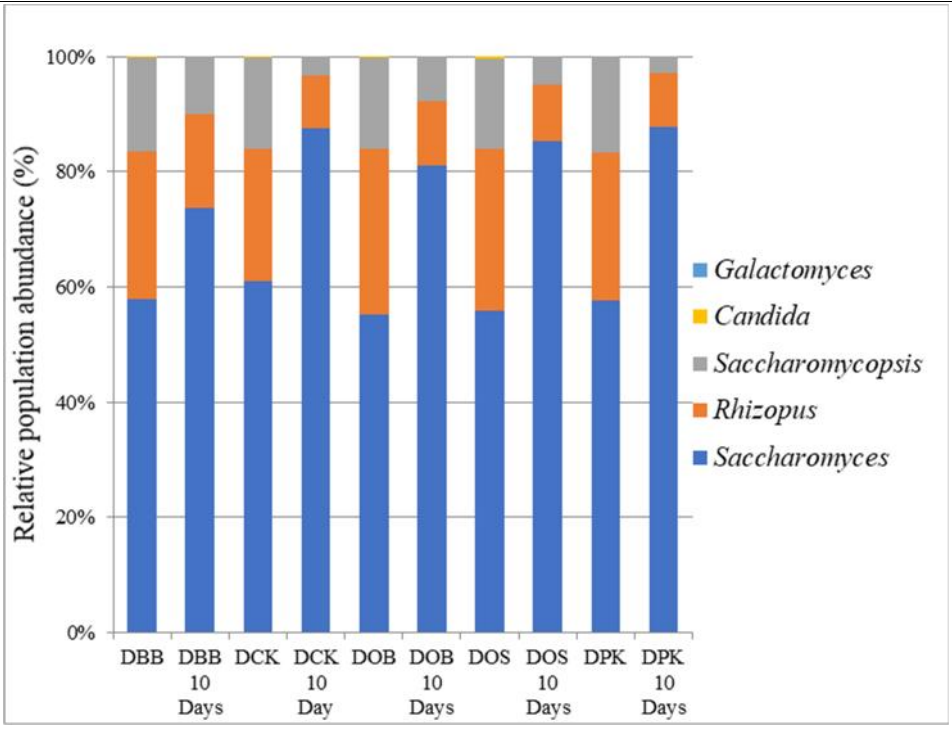

Figure 3: Fungal composition (OTUs at the genus level based on 28S amplicon sequencing) of the five starters and the corresponding microbial communities after 10 days of fermentation (labelled "10 Days").

DBB, DCK, DOB, DOS, and DPK are the five traditional Cambodian starters.

Carbohydrate consumption and ethanol production during the traditional fermentation with five various starters

Sugars and ethanol were measured every 24 hours. The profiles of sugar consumption and ethanol production are shown in **Figure 5**. In rice wine production, the immersion of rice in water and the steam cooking steps are believed to play a role in the breaking down of the structure, to accelerate starch gelatinization and to sterilize rice from microbial agents. According to the results of the microbial community above, *Rhizopus* spp. was associated in the five starters. The presence of this species illustrated that amylolytic enzymes were produced during the brewing process. *R. oryzae* was reported as a strong amylase producer frequently found in amylolytic

fermentation starters for rice wine (Dung et al., 2007; O'Brien and Wang, 2008; Thanh et al., 2008; G. Xie et al., 2007), and was found frequently during traditional fermentation process of Hong Qu glutinous rice wine (X. C. Lv et al., 2015).

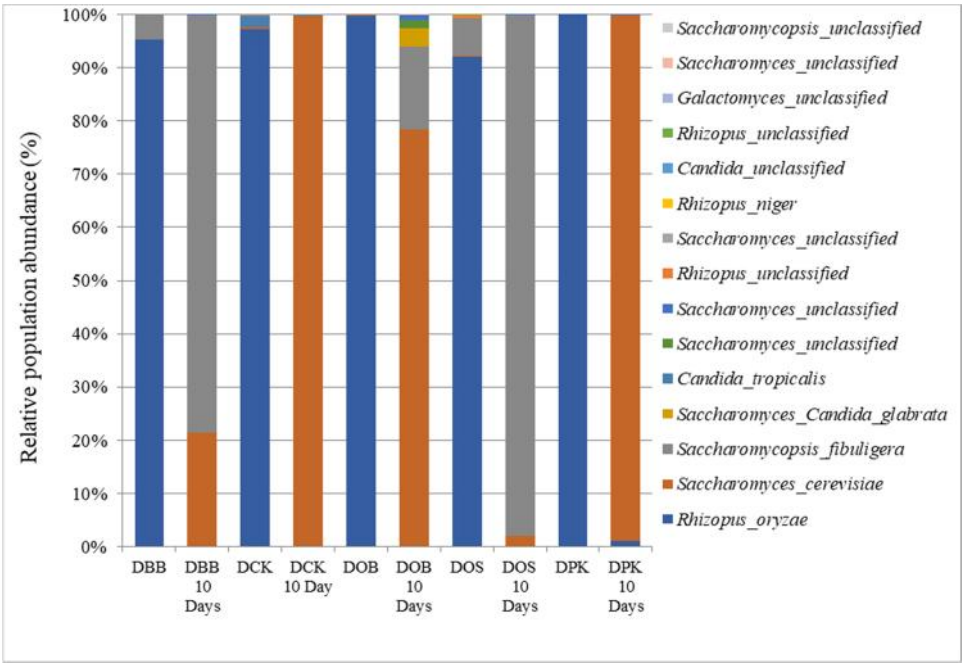

Figure 4: Fungal composition (OTUs at the species level based on 28S amplicon sequencing) of the five starters and the corresponding microbial communities after 10 days of fermentation (labelled "10 Days").

DBB, DCK, DOB, DOS, and DPK are the five traditional Cambodian starters. Amylolytic enzymes hydrolyse starch in smaller molecules. In this work, maltotriose and maltose were detected but in small quantities. The profiles of maltotriose and maltose are shown in **Figure 5A** and **5B**, respectively. The concentrations in these two products reached maximal values at the third day due to the solid-state fermentation (steamed red rice with a moisture content approximately of 62%). Some liquid production was observed during this solid-state fermentation. Water was added to induce the alcoholic fermentation.

At the end of fermentation, maltotriose and maltose were still present and gave rice wine a sweet taste. Surprisingly, glucose was much more produced during this brewing process **(Figure 3-5C)**. The highest concentration in glucose reached a maximal value (from 300 to 550 mg/L) at the third day in all fermentation cases.

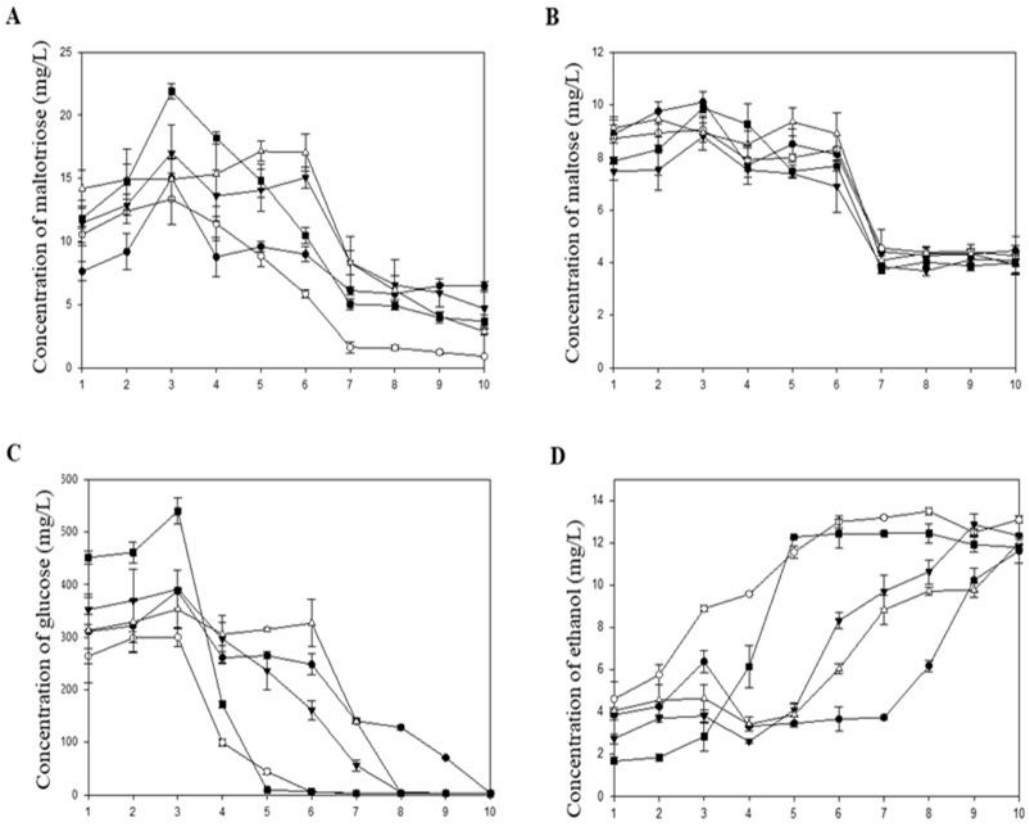

Figure 5: Kinetic of carbohydrate consumption and ethanol production during fermentation based on five microbial starters.

(A) Maltotriose, (B) Maltose, (C) Glucose, and (D) Ethanol concentration. ($^{\Delta}$DBB, $^{\bullet}$DCK, ^{o}DOB, ▲ DOS, and ■ DPK).

After eight days of fermentation, there was no more glucose except in the sample of the DCK starter which ended the fermentation at the tenth day. The results highlighted

that there has been a production and a consumption of sugar simultaneously during this brewing. This was due to the presence of amylolytic filamentous fungi and yeasts present in all ferment starters. The evolution of glucose consumption was correlated with the ethanol production. Since the first day of brewing, ethanol was produced in slight concentration (ranging from 2 to 5% v/v). At the fourth day, the concentration slightly decreased because water was added to boost the alcoholic fermentation. It has been observed that the brewing with the DOB and DPK starters occurred faster. Glucose was totally consumed after 6 days and the ethanol production was maximal at the same time. This was due to the predominance of *Saccharomyces cerevisiae* in these starters. However, the final ethanol concentrations were almost similar (between 11.6 and 13 % v/v). The final concentration in ethanol at the end of fermentation in this study was similar to the study of Liu et al., (2014).

Volatile compounds produced by the starters

Twenty-five aromatic compounds were identified by matching to MS library spectra and matching calculated retention time index (RI) values to literature values. The fermentation of red rice wine was made in three replicates in the same conditions with the five starters. The analysis of aromatic compounds was performed in biological triplicate using SPME-GCMS. SPME has been widely used as a method to determine volatile aromatic compounds in rice wine (Ha et al., 2014; Jung et al., 2014; Xiao et al., 2014). A previous study reported that DVB/CAR/PDMS fibre was applicable to the detection of a wide range of aromas in beer, which is also a cereal based beverage (Rodrigues et al., 2008). As results, **Table 3** showed the twenty-five compounds identified including esters, alcohols, acids, aldehydes and ketones. Amongst the quantified volatile compounds, the most abundant group was alcohols (about 93% of the total aromatic compounds). As shown in **Table 3**, 2-methylbutan-1-ol, 3-methylbutan-1-ol, butane-2,3-diol and 2-phenylethan-1-ol were the main volatile compounds. The 3-methylbutan-1-ol was found to be the dominant volatile compound

in the different samples (around 54% w/v). The 2-methylpropanol, with a pleasant whiskey flavour, was detected in higher concentrations in the DBB rice wine sample (5976.46 μg/L) and in the DOB sample (5076.98 μg/L) while the concentration was lower in the DOS and DCK samples. Another floral aromatic compound, 2-phenylethan-1-ol, was also found as the third major compound in the five rice wines. Rice wine fermented with DPK showed the highest 2-phenylethan-1-ol production amongst those rice wines with a concentration of 3624.76 μg/L while the lowest concentration was found in the DCK sample with only 1607.37 μg/L. Butan-2,3-diol was described as a fruity aroma and was also identified in each rice wine. There were numerous by-products stemming from alcohol fermentation including this compound. It was considered the second most abundant potential source of aroma. The only aldehyde identified and quantified was acetaldehyde. The 2-phenylethylacetate was only found in the rice wine fermented by starter DBB. It is a colourless liquid with a rose flavour that contributes to 'rose,' 'honey,' 'fruity' and 'flowery' aroma nuances (Swiegers et al., 2005). Only three ketones were identified in this study including octan-2-one, 3-hydroxybutan-2-one and acetophenone.

Correlation between volatile compound and bacteria and fungi species

The correlation between the volatile compound and bacteria and fungi species presented in each dried starter is shown in **Table 4**. Cytoscape Network software was used for visualizing the interaction and correlation **(Figure 6)**. Only the correlation coefficient significant at least at 0.05 level were discussed in this part. The correlation coefficient indicated a very strong relation (from 0.882 to 1). The complexity of variety of microbial community have generated intricate and specific aromatic profiles. Relatively high and significant correlations with volatile compound produced were observed with the presence of various strains including mostly *Weissella* genus; *Weisella cibaria, Weissella paramesenteroides, Weisella confusa, Weisella*

unclassified, Acetobacteraceae liquefaciens, Lactobacillus plantarum, Lactobacillus fermentum, Lactobacillaceae unclassified, Pediococcus sp. MMZ60A, Pediococcus unclassified, Leuconostoc galicum, Lactococcus lactis, Streptococcus GV636515 and *Saccharomycopsis fibuligera.* Phenyl ethylalcohol, a pleasant floral odor; benzyl alcohol, mild pleasant aromatic odour, were strongly correlate with the most of *Weissella* and *Pediococcus* genus. Phenyl ethylacetate was found to be perfect correlated with only *Saccharomycopsis fibuligera.* Negative relation of octanone were observed with the presence of *Lactococcus lactis, Leuconostoc garlicum, Lactobacillaceae unclassified,* ethyl acetate with *Weisells unclassified1* and butanol with *Acetobacteraceae liquefaciens.*

Table 3: Correlation between the volatile compounds produced by each dried starter and bacteria and fungi species presented in each starter.

Strains	Compounds	Pearson Correlation coefficient	p-value
Saccharomycopsis_fibuligera	Phenyl ethylacetate	1.000	0.000
Lactobacillus_plantarum	Acetic acid	0.997	0.000
Pediococcus_pentosaceus	pentanol	0.994	0.001
Enterococcus_durans	pentanol	0.994	0.001
Weissella_unclassified3	pentanol	0.994	0.001
Pediococcus sp. MMZ60A	pentanol	0.993	0.001
Weissella_paramesenteroides	pentanol	0.993	0.001
Streptococcus_GV636515	Acetic acid	0.993	0.001
Pediococcus_unclassified	pentanol	0.993	0.001
Leuconostoc_garlicum	Acetic acid	0.992	0.001
Acetobacteraceae_liquefaciens	Acetic acid	0.990	0.001
Lactobacillus_fermentum	Acetic acid	0.989	0.001
Lactococcus_lactis_subsp_cremoris	Ethoxyl propanol	0.988	0.001
Lactobacillaceae_unclassified	Ethoxyl propanol	0.987	0.002
Lactococcus_lactis_subsp_cremoris	Acetic acid	0.966	0.007
Lactobacillaceae_unclassified	Acetic acid	0.965	0.008
Weissella_cibaria	hexanol	0.963	0.008
Weissella_unclassified4	hexanol	0.961	0.009
Leuconostoc_garlicum	Methoxyphenol	0.960	0.010
Lactobacillus_plantarum	Ethoxyl propanol	0.958	0.010
Weissella_unclassified1	Phenyl ethylalcohol	0.953	0.012
Streptococcus_GV636515	Ethoxyl propanol	0.952	0.013
Weissella_unclassified5	Octanoic acid	0.952	0.013
Lactobacillus_fermentum	Ethoxyl propanol	0.951	0.013
Acetobacteraceae_liquefaciens	Ethoxyl propanol	0.949	0.014
Leuconostoc_garlicum	Ethoxyl propanol	0.948	0.014
Lactococcus_lactis_subsp_cremoris	methoxyphenol	0.948	0.014
Lactobacillus_plantarum	methoxyphenol	0.939	0.018
Weissella_unclassified1	Pentanol	0.933	0.021
Lactococcus_lactis_subsp_cremoris	Octanone	-0.926	0.024
Lactobacillaceae_unclassified	Methoxyphenol	0.922	0.026
Pediococcus_pentosaceus	Ethyl oleate	0.921	0.026
Pediococcus_unclassified	Ethyl oleate	0.921	0.026
Enterococcus_durans	Ethyl oleate	0.921	0.026
Weissella_paramesenteroides	Ethyl oleate	0.920	0.027
Pediococcus sp. MMZ60A	Ethyl oleate	0.919	0.027
Pediococcus_unclassified	Phenylethyl alcohol	0.919	0.027
Weissella_unclassified3	Ethyl oleate	0.919	0.027
Weissella_confusa	Hydroxy butanone	0.919	0.027
Weissella_unclassified3	Phenylethyl alcohol	0.918	0.028
Pediococcus_pentosaceus	Phenylethyl alcohol	0.917	0.029
Enterococcus_durans	Phenylethyl alcohol	0.917	0.029
Weissella_paramesenteroides	Phenylethyl alcohol	0.915	0.029
Pediococcus_EU157914	Phenylethyl alcohol	0.914	0.030
Streptococcus_GV636515	methoxyphenol	0.913	0.030
Weissella_unclassified1	Ethyl acetate	-0.911	0.031
Weissella_unclassified1	Acetaldehyde	0.908	0.033
Lactobacillus_fermentum	methoxyphenol	0.903	0.036
Lactobacillaceae_unclassified	octanone	-0.902	0.036
Acetobacteraceae_liquefaciens	methoxyphenol	0.901	0.037
Weissella_unclassified5	benzylalcohol	0.885	0.046
Leuconostoc_garlicum	octanone	-0.882	0.048
Pediococcus_unclassified	Acetaldehyde	0.882	0.048
Acetobacteraceae_liquefaciens	Butanol	-0.878	0.050

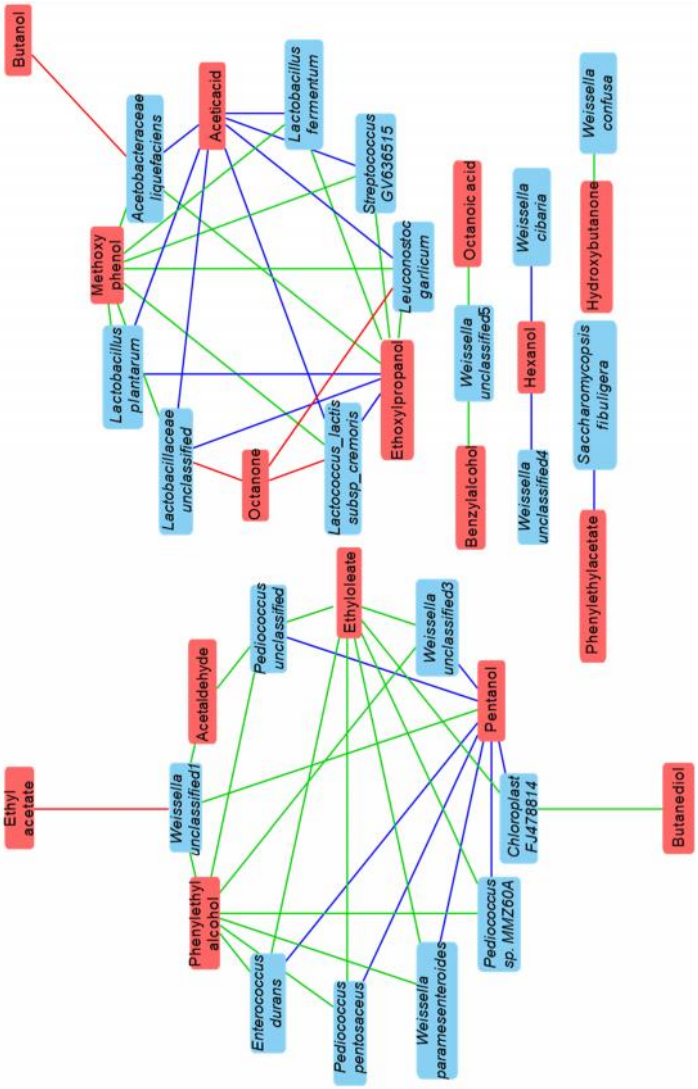

Figure 6: Correlation network between volatile metabolites and microbial starters (based on Cytoscape software). The red boxes represent volatile metabolites and blue boxes correspond to microbial strains that are correlated with this metabolite. The blue line represents the positive correlation with a level of significance of 0.01, the green line represents the positive correlation with a level of significance of 0.05 and the red line represents the negative correlation with a level of significance of 0.05.

Table 4: volatile compounds (µg/L) identified in the Cambodian traditional dark red rice wine after 10 days

Compounds	RI	RI	DBB		DCK		DOB		DOS		DPK	
	Cal	Lit[a]	Means	SD	Means	SD	Means	SD	Means	SD	Means	SD
Esters												
Ethyl lactate	1360	1358	UD		292.43	6.64	483.90	57.55	241.00	13.39	353.35	7.51
Ethyl acetate	901	898	12.41	0.91	17.50	0.09	28.14	2.78	17.62	0.90	UD	
2-Phenylethyl acetate	1828	1837	58.69	11.14	UD		UD		UD		UD	
Ethyl oleate	2492	2489	UD		UD		27.37	1.82	10.79	0.95	68.65	4.33
∑			71.11		309.93		539.40		269.41		422.00	
Alcohols												
Propan-1-ol	1049	1037	1176.71	135.52	815.01	56.50	1762.73	15.93	731.38	49.23	423.41	44.40
2-Methylpropan-1-ol	1072	1099	5976.46	378.32	3668.25	557.68	5076.98	850.15	3507.64	308.37	4399.94	111.80
Butan-1-ol	1184	1145	56.26	5.40	34.50	2.34	63.50	2.85	49.75	5.39	63.81	3.73
3-Methylbutan-1-ol	1237	1205	13781.86	1588.87	10077.08	1385.76	17546.66	1472.95	10235.16	694.25	16320.50	2265.41
Pentan-1-ol	1269	1255	17.11	1.10	16.49	1.58	17.78	1.97	16.73	1.16	26.73	1.78
Hexan-1-ol	1362	1360	87.76	8.32	UD		75.48	3.47	54.85	5.38	UD	
3-Ethoxypropan-1-ol	1374	1376	20.43	3.03	28.25	0.01	22.39	1.70	21.46	2.76	22.96	1.04
Heptan-1-ol	1460	1467	14.93	1.40	15.93	1.29	18.04	0.27	10.72	0.11	18.97	0.02
Butane-2,3-diol	1548	1523	1067.14	104.64	1483.15	161.96	1716.60	127.39	1146.23	61.66	2230.13	168.35
Octan-1-ol	1559	1553	UD		0.00	0.00	34.63	2.80	36.97	4.23	16.87	1.03
2-methoxyphenol	1877	1875	40.45	5.45	95.01	0.97	56.66	2.43	23.14	0.99	38.69	2.11
Phenylmethanol	1891	1865	8.80	0.77	17.30	1.01	23.38	1.34	8.24	0.88	15.71	1.66
2-Phenylethan-1-ol	1928	1925	2302.53	315.41	1607.37	29.65	2228.61	27.35	2318.04	29.65	3624.76	140.48
∑			24550.45		17858.35		28643.43		18160.30		27202.48	
Acids												
Acetic acid	1470	1450	633.46	31.22	1854.84	8.25	766.58	55.86	638.96	82.99	546.93	35.79
2-methylpropanoic acid	1584	1563	UD		35.35	2.82	UD		83.66	0.06	91.38	8.28
Octanoic acid	2088	2083	18.07	1.75	48.59	5.49	188.59	9.32	10.48	0.38	23.23	2.96
Butanedioic acid	1680	1619	63.72	2.64	67.14	1.24	87.83	5.34	20.88	0.66	13.95	0.29
∑			715.25		2005.92		1043.00		753.98		675.49	
Aldehydes and ketones												
Acetaldehyde	691	690	116.91	11.99	72.84	4.93	73.66	1.08	207.14	3.45	339.28	44.12
Octan-2-one	1295	1285	40.45	4.51	24.08	2.24	32.00	2.72	35.94	1.32	32.70	0.29
3-Hydroxybutan-1-one	1310	1295	84.20	5.46	33.14	1.60	34.72	0.81	106.32	7.04	56.10	0.69
Acetophenone	1664	1645	18.98	4.03	13.33	0.53	UD		UD		UD	
∑			260.54		143.40		140.38		349.40		428.07	
Total aroma profile			25597.35		20317.59		30366.22		19533.09		28728.04	

UD: Under the detection threshold, [a]: Literature source http://www.pherobase.com/

DISCUSSION

This study represents the first attempt using rRNA pyrosequencing to investigate the microbiota in five different Cambodian traditional dried starters, and to examine the changes of microbial composition after 10 days of fermentation. It has been reported that the microbiota composition of rice wine starter was highly variable (Sujaya et al., 2001; Thanh et al., 2008). The results observed in this study were in agreement with the previous findings of Lv et al., (2013) and Ramos et al., (2011) at the level of lactic acid bacteria (LAB). The prevalence of LAB in fermented food was commonly due to their ability to tolerate low pH values (Abriouel et al., 2006). This is the reason that potential foodborne pathogens were not detected after having achieved the traditional rice wine fermentation process. The composition of LAB in the starters applied to the production of alcoholic beverages was also investigated by Thanh and his team (2008). Their results showed that *P. pentosaceus, L. plantarum, L. brevis, W. confusa* and *W. paramesenteroides* were detected in Vietnamese starters using a 16S rRNA gene-based PCR-based denaturing gradient gel electrophoresis. However, only the bacterial population that represents at least 1% of the total community would probably be detected by DGGE (Weisburg et al., 1991). Thus, the meta-genomic analysis is a useful tool to investigate the composition of microbial communities since it is capable to detect lower populations. Basically, a spontaneous cereal-based fermentation is induced by the combination of yeasts, fungi and lactic acid bacteria (Blandino et al., 2003). The study of Nout and Sarkar (1999) have shown that the growth of yeasts in fermented food is favoured by the acidification caused by bacteria. Another study revealed that Saccharomyces cerevisiae adjust its metabolism by secreting a serial metabolite, notably amino acid, allowing the survival of LAB (Ponomarova et al., 2017).

The presence of LAB in cereal fermentation is probably crucial because beside producing lactic acid, LAB is likely to contribute production of other flavour compounds (Mukisa et al., 2017). Environmental stress, particularly acid stress; induced the formation of specific aromatic compounds during the lactic acid fermentation (De Angelis et al., 2001; Serrazanetti et al., 2011, 2009). Therefore, the aroma type and its concentration might be determined by the substrate composition, the starter culture and the environmental conditions of the process. The taxonomic analysis has shown a complex bacterial community in the Cambodian dried starters, even after the fermentation stage with red rice as a raw material. Most species were identified as lactic acid bacteria but they varied in different proportions. The genera Lactobacillus, Leuconostoc, Weissella and Pediococcus were found on the grains' surface and in the surrounding environment. This is the fact that they are found with fungal strains in fermented cereal based food (Guyot, 2012). LAB are also seen as favourable microorganisms associated with cereal based beverages since it has been shown that they improve protein digestibility, increase nutritional bioavailability and enhance organoleptic quality (Luana et al., 2014). Based on the traditional brewing, the variety of the starters is an important factor influencing both the rice wine flavour and quality. The growth of LAB species during rice wine brewing might affect the growth of yeasts and filamentous fungi, which also contributes to the flavour of rice wine (Lv et al., 2013). To notice that the locally produced dried starters by rice wine producers could be different based on their individual methods and specific ingredients from one to another region. This variation might therefore affect the starters' quality in terms of final composition of the microbial consortia found in the starters.

There were changes in fungal diversity after 10 days of fermentation, at both levels of filamentous fungi and yeast species. This might be due to the predominance of species in starter, the decreasing pH induced by the LAB and the protocol of starter

preparation. The microbial composition of starters varied according to the regions where they were produced and was influenced by the environment and the material used. According to the study of Yamamoto and Matsumoto (2011), traditional dried starters have widely been used for rice fermentation in Cambodia. Herbs and spices were used as ingredients for the production of dried starters including ginger, chili, pepper, cloves etc. Mixing cultures with spices and oriental herbs were believed to prevent the growth of unfavourable microorganisms and to enhance the synthesis of interesting aromas. Many studies reported various fungi and bacterial species in starters (Kofi E Aidoo et al., 2006; Dung et al., 2006; Jeyaram et al., 2008b; Thanh et al., 2008). The study of Dung et al. (2005) focused on the effect of each oriental ingredient frequently added to dried starters in Vietnam. This study revealed that various herbs and spices have a great impact on biomass and the yeasts during the fermentation. In Cambodia, both dried starters and rice wine preparations are done in an opened environment. This leads to increase the microbial diversity. This process must also ensure a good organoleptic quality of the final product. The flavour profile is the most important characteristic of rice wine and can be affected by the consortium of microorganisms used. It has been shown that the flavour of rice wine could be changed and increased when the fermentation process is performed by non-*Saccharomyces* species (Medina et al., 2013). The behaviour of *R. oryzae* was observed and its ability to produce volatile compounds during fermentation such as ethanol, 2-methylpropanol and 3-methylbutanol was highlighted (Bramorski et al., 1998; Christen et al., 2000). These two last compounds were the major aromatic molecules produced by the five starters (Table 3). Each dried starter contained *Saccharomyces cerevisiae* and *Saccharomycopsis fibuligera.* However, the yeast species which was prevalent in the dried starters became dominant after 10 days of fermentation. For example, in the cases of the dried starters DBB and DOS, *S. fibuligera* got dominant (final proportion of 78.39% and 97.92% of OTUs, respectively) while this species was found in high proportions in the original ferment

starters. In the starters DCK, DOB and DPK, *S. cerevisiae* was the only fermenting species in OTUs' proportions of 99%, 78% and 98%, respectively. The DCK and DPK starters containing only *S. cerevisiae* as the prevalent species performed the fermentation slower than the other two dried starters which contained *S. fibuligera* alone or in combination with another fermenting species. This performance was observed due to the glucose consumption and ethanol production speed (**Figure 5**). However, the final concentrations in ethanol were not significantly different after 10 days of fermentation (between 11.6 and 13 % v/v). The presence of *S. cerevisiae* and *S. fibuligera* was in good agreement with the study of Lv et al. (2013) which studied on yeast diversity in Chinese traditional starters. This study provided evidence that each microorganism plays a role in the consortium, and therefore affects the final quality of the product derived from the fermentation process. Similar study of Sha et al., (2017) revealed that *Marcha* and *Thiat*; ferment starter in India and Nepal, composed different fungal communities. *S. cerevisiae* produces small quantities of 3-methylbutan-1-ol under fermentative condition at low pH. *S. cerevisiae* generate L-leucine via pyruvate metabolism, and 3-methylbutan-1-ol is generated via the L-leucine degradation III pathway. This compound provides wine with a malt-like odour. In Chinese rice wine (Xiao et al., 2014), guava wine (Pino and Queris, 2011) and cherry wine (Dung et al., 2005; Niu et al., 2011), esters were found to be the major volatile compounds. Acetate esters and ethyl esters of fatty acids are formed by the reaction of an organic acid with alcohol during the fermentation, leading to fruity aromas in wine (Villamor and Ross, 2013). However, in this study, the alcohol group was predominant. It could be due to the absence of reactions between carboxylic acids and alcohols. Another reason is because of freshly harvesting and analysing SPME-GCMS quite immediately after fermentation to see the different flavour compound produced by the communities. In general, most flavour compounds, especially esters in rice wine, are principally produced after fermentation (Wang et al., 2014). The aromas' types and their concentrations might be influenced once more by the substrate

composition, the starter culture, the environmental conditions and the process applied. Some species presented in small quantity in the community still have strong correlation with volatile compounds. It was found that *Weissella, Pediococcus* and *Lactobacillus* genus has most mutually related with flavour compounds. Go through to the bacterial community of DOB starter, *L. fermentum* and *L. plantarum* were found as dominant at the end of fermentation while the volatile compound was hugely produced. Lactobacillus is an important genus involved in grape fermentation. *L. Plantarum* is certainly found frequently on grape and in wine and is often involved in spontaneous malolactic fermentation. Recently, some researchers have revealed that *L. plantarum* species shows a different enzymatic profile to other LAB species, which could play an important role in the wine aroma profile (Iorizzo et al., 2016; Lerm et al., 2011; Swiegers et al., 2005). The interaction between LAB and yeasts has been known to enhance the growth of either group of microbes (Mugula et al., 2003; Omemu et al., 2007) and to build up the alternative flavour production (Mukisa et al., 2017).

This study highlighted the variable pattern structure of microbiota in the spontaneous red rice wine fermentation. The variable categories and concentrations of the flavour compounds were intensely affected by the nature of these microbial communities. Competitive metabolic interactions among species often play a critical role in the structure and the functions of multispecies communities. However, metabolic interactions still play an important role in regulating microbial activities and in maintaining the diversity in microbial communities during the brewing process itself. The results presented here fully enrich our understanding of the microbial community exploited in rice wine brewing and the corresponding aromatic profiles. Further studies should be performed to understand the interactions between LAB, yeasts and molds to define the most important factors contributing to the final flavour of rice wine.

Chapter 3.2: Engineering synthetic microbial communities through selective biofilm cultivation device for the production of fermented beverages

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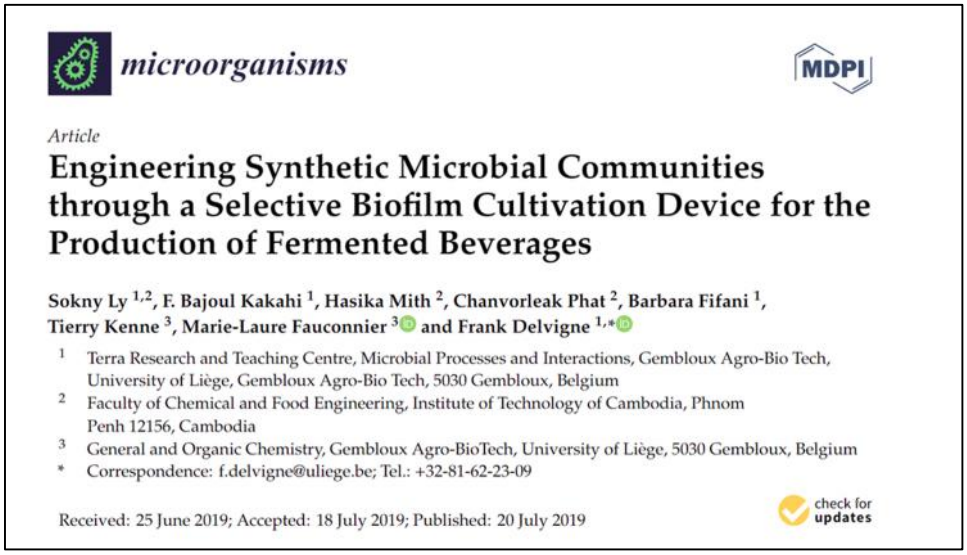

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Engineering Synthetic Microbial Communities through a Selective Biofilm Cultivation Device for the Production of Fermented Beverages

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ABSTRACT: Production of Cambodian rice wine involves complex microbial consortia. Indeed, previous studies focused on traditional microbial starters used for this product revealed that three microbial strains with complementary metabolic activities are required for an effective fermentation, i.e. filamentous fungi (*Rhizopus oryzae)*, yeast (*Saccharomyces cerevisiae*) and lactic acid bacteria (*Lactobacillus plantarum*). Modulating the ratio between these three key players led to significant differences not only in terms of ethanol and organic acids production, but also on the profile of volatile compounds, by comparison with natural communities. However, we observed that using an equal ratio of spores/cells of the three microbial strains during inoculation led to flavour profile and ethanol yield close to that obtained through the use of natural communities. Compartmentalization of metabolic tasks through the use of biofilm cultivation device allows further improvement of the whole fermentation process, notably by increasing the amount of key component of the aroma profile of the fermented beverage (i.e., mainly phenylethyl alcohol, isobutyl alcohol, isoamyl alcohol and 2-methyl-butanol) and reducing the amount of off-flavour compound. This study is a step forward in our understanding of microbial interkingdom interactions with strong application potential in food biotechnology.

KEYWORDS: microbial interactions; volatolomic; biofilm; alcoholic fermentation

INTRODUCTION

Traditional fermented foods have an important place in the food culture of human society worldwide, as the fermentation enhances the shelf life, texture, taste, aroma and nutritional value of the foods (Uzogara et al., 1990). In Cambodia, several fermented products have been developed from rice grain such as rice vinegar, fermented rice (*Tampè*) and rice wine (*Sra sor*). Among those, rice wine is the most common application of rice fermentation while its production is still made under traditional practices. Rice wine producers regularly met the problems of low yield and inconsistent quality in terms of taste and flavour. These traditional processes lack of

research and optimization in the field of food biotechnology. The principle of rice wine production consists of saccharification of steamed starchy source by fungi under solid state fermentation and alcoholic fermentation by yeasts under submerged fermentation (Blandino et al., 2003; Dung et al., 2007; Sujaya et al., 2004). The nature of microbial communities in Cambodian traditional starters, their interactions and their contributions to the aromas synthesis during fermentation are still widely unknown. Microbial diversities in ferment starters were studied by many researchers (Chao et al., 2013; Ercolini, 2004; Jeyaram et al., 2008a; Luangkhlaypho et al., 2014; Lv et al., 2012b; X.-C. C. Lv et al., 2015; Ly et al., 2018; Thanh et al., 2008; Wang et al., 2015). However, there is a very limited number of studies to exploit the interaction between these three groups of microorganisms and their effect on rice wine quality during fermentation process. Liu et al. (2017) pointed out that wine fermentation is not a single-species process, and the role of the different microbial wine-related species in wine production is in the attention of worldwide research. Volatile compounds of wine produced by mixed cultures of *Saccharomyces cerevisia*e and non-Saccharomyces strains were significantly different from those made by mono-culture (Sadoudi et al., 2012). This indicates the important metabolic interaction between yeast strains during fermentation. As mentioned above, not only yeast but also filamentous fungi and LAB (Lactic acid bacteria) are involved in rice wine production, and it would be very informative and helpful to investigate the impact of these three groups on volatile compound produced in rice wine.

Filamentous fungi in submerged and semi-solid conditions in rice wine production can lead to either an increase of broth viscosity or a decrease of nutrient diffusion rate due to their different morphologies, ranging from dispersed filaments to pellets (Papagianni, 2004). Large-scale applications are limited due to the appearance of oxygen and nutrient gradients inside the solid mass (Zune et al., 2015). Optimal management of microbial communities can be achieved through the design of cultivation media promoting metabolic interactions (Klitgord and Segrè, 2010) or by

the design of alternative cultivation device enhancing the spatial organization of the community (Hays et al., 2015). The design of biofilm cultivation device has been previously optimized to improve the natural binding of fungal biomass on inert surface (i.e., metal wire gauze packing). This alternative cultivation device has been developed for the production and purification of hydrophobin HFBII from filamentous fungi *Trichoderma reesei* (Khalesi et al., 2014) and for the production of recombinant glucoamylase by *Aspergillus oryzae* (Zune et al., 2015). In both cases, the fungal system displayed strong attachment on the metal packing, without any significant growth in the liquid phase. On the opposite, yeast (Vandermies et al., 2018) and bacterial (Zune et al., 2016, 2014) systems exhibited lower attachment in similar device with a significant proliferation in the liquid phase. Thus, based on the differential attachment on the inert surface, biofilm cultivation device could be used for promoting structuration of microbial species within communities involving fungi, yeast and bacteria.

MATERIALS AND METHODS

Strain and medium preparation

Yeast strain *Saccharomyces cerevisiae*, filamentous fungi *Rhizopus oryzae*, and lactic acid bacteria *Lactobacillus plantarum* were isolated from Cambodian traditional dried ferment starter (*Dombea*) and used in this study. These three strains were isolated at different stage of rice wine fermentation process. *R. oryzae* was isolated after inoculated cooked rice with ferment starter (saccharification step) while *S. cerevisiae* and *L. plantarum* were isolated during alcoholic fermentation. *R. oryzae* was incubated in Dichlorane Rose Bengal Chloramphenicol medium and grown for 72 hours followed by harvesting spores and stored in 30% glycerol. *S. cerevisiae* and *L. plantarum* were inoculated in yeast extract peptone dextrose and MRS broth; respectively, for 24 hours and stored in 30% glycerol at -80 ^{o}C for further use. Pigmented rice (Red rice) and artificial liquid rice media were used and studied.

Fermentation based on red rice

A laboratory-scale fermentation of red rice was adapted for mimicking traditional process used by local rice wine producers. Briefly, 100 g of red rice were soaked in distilled water for 3 h. The soaking water was discarded and a volume of 100mL of distilled water was then added and steamed in an autoclave at 120 ºC for 20min. The gelatinized rice paste was cooled down to room temperature and further inoculated and mixed with 2% of traditional dried starter (purchased from local producer) before being incubated at 30 ºC. After a solid-state aerobic fungal fermentation of 3 days, an additional volume of 100mL of sterilized water was added to boost the alcoholic fermentation for other 7 days more in the same flask. The fermented rice mashes were homogenized and the sampling was made every 24 h for 10 days.

Fermentation based on synthetic liquid medium

In order to investigate the interaction between these three potential culture strains, artificial liquid rice media were created based on major compounds in rice (Sompong et al., 2011) and used in packing system flask. Metal packing (wire gauze, 316L stainless steel) used in the study of Zune and his team were adapted in order to fit in the middle of 250 mL Erlenmeyer flask with 100 mL of media (see **Figure 4B** for a scheme of the device) (Zune and Toye, 2013). Additionally, flasks without metal packing have been considered as control, i.e. considered to be equivalent to traditional submerged fermentation. The composition of artificial rice media was created according to the potential rice components. The mixture of soluble starch 20g/L, arginine 0.196g/L, alanine 0.151 g/L, leucine 0.214 g/L, valine 0.151 g/L, phenylalanine 0.133 g/L, glutamic acid 0.526 g/L, aspartic acid 0.242 g/L, CaCl2 0.124 g/L, $FeSO_4$ 0.0012 g/L, $MgCl_2$ 0.0028 g/L, $CuSO_4$ 0.0024 g/L, $MnSO_4$ 0.0051 g/L, K_2HPO_4 1.584 g/L, and KH_2PO_4 0.58 g/L was adjusted pH to 6.5 with potassium phosphate buffer. All inocula were added into liquid phase and started at the appropriate concentration spores or CFU/mL.

Aromatic compounds analysis by HS-SPME-GC-MS

Analysis of the volatile compounds was performed based on Head-Space-Solid-Phase Microextraction (HS-SPME) followed by Gas-Chromatography-Mass-Spectrophotometry GC-MS analysis. The sample was extracted using a 50/30 µm Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) fibre (Supelco, Inc., Bellefonte, PA). Each liquid sample (5 mL) was placed in a 20 mL SPME glass vial together with 30% w/v of sodium chloride and 1 µL of the internal standard 2-octanol (0.4095 mg/L in absolute methanol). The vial was tightly capped, shake and left to equilibrate for 30 min at 60°C and then fibre was exposed to the headspace for 30 min. The fibre was introduced into the injection port of the GC-MS system (at 250°C for 10 min) and the analysts extracted from the fibre were thermally desorbed. The analysis was done in the splitless mode using helium at a total flow rate of 50 mL/min. The identification of the extracted compounds was performed in a Shimadzu GC-2010plus with Rtx-5MS capillary column. The column carrier gas was helium at a flow rate of 1.5 mL/min. The mass detector operated in the electron impact mode was relative to the tuning result in a range from 35 to 550 m/z, and the ion source temperature was set at 230°C. The oven temperature was held at 35°C for 1min, raised at 6°C /min to 155°C, then raised to 250°C at a rate of 10°C / min, and held at 250°C for 20min. The aromatic components were identified by comparison of their Retention Indices with data reported in the literature and their mass spectra the NIST 11 data base (matching quality higher than 90%). The Retention Indices (RI) of unknown compound were calculated by the retention time of a series of alkanes (C5-C35). Semi-quantitative analysis of the volatile compounds was performed using octan-2-ol as the internal standard. The results were reported on the basis of a mean value from two biological and analytical replicates

Sugar and ethanol analysis by HPLC

The concentrations of glucose, ethanol, acetic and lactic acid were determined using High-Performance-Liquid-Chromatography coupled with Refractive index detector (RID-HPLC) (Shimadzu LC20A). A volume of 5 µL of sample was injected, in duplicate, through a RezexROA-Organic Acid column (300×7.8mm) with 5mM H_2SO_4 as mobile phase at a flow rate of 0.6 mL/min at 60 ^oC.

Microbiological analysis

Yeast and LAB growth were followed by selective plate count with dechlorane rose bengal with 0.01% chloramphenicol (Merck) and MRS agar with 0.01 % cycloheximide (Merck), respectively. Biomass attached on packing was measured after drying for 24 hours at 105 ^oC followed by subtracted the mass of metal packing.

Statistical analysis

ANOVA of chemical and volatile compound analysis was done for the different fermentation treatments. Mean rating and Least Significant Differences (LSD) for each treatment were calculated from each analysis of variance with Minitab 18. Principal component analysis (PCA) was performed to establish the relations of aroma compound between samples.

RESULTS

Comparison of natural and synthetic community for red rice wine fermentation

Ethanol and organic acid production during red rice wine processing

Previous study reported that Cambodian traditional ferment starter (natural community) contains *Rhizopus oryzae* as the main filamentous fungi, *Saccharomyces cerevisiae* as the main fermenting yeast and *Lactobacillus plantarum* as the main lactic acid bacteria. Additionally, the presence/co-occurrence of these three microbial strains was associated with the generation of major flavour compounds. In this study, red rice wine fermentation kinetics were investigated for both culture with natural community and synthetic community made of *R. oryzae*, *S. cerevisiae* and *L. plantarum*. Sugar, ethanol and organic acids play an important role in wine tastes and quality; accordingly, these parameters were investigated during the process (**Figure 1**). The time evolution of ethanol and organic acid production was correlated with the consumption of glucose. Maltose and glucose were the main reducing sugar detected in this study (Supplementary **Figure S1**). Surprisingly, synthetic communities comprising high yeast inoculant (i.e. *Ro-Sc* 1:10 and *Ro-Sc-Lp* 1:10:1) haven't provided to the highest ethanol production. This type of observation have been made previously for Chinese fermented beverages (Yang et al., 2014). According to Table 1, the significant highest ethanol productions were observed when using natural community, followed by the community *Ro-Sc-Lp* 1:1:1. Whereas natural community gave the highest ethanol yield, it also led to the accumulation of acetic and lactic acid level, two compounds being considered as off-flavour compounds for wine.

Comparative analysis of the impact of natural and synthetic communities on the flavour profile during red rice wine fermentation

Flavour and aroma profiles are important factors responsible for the organoleptic quality of wine. In this study, flavours compounds produced during fermentation process were analysed to investigate and evaluate the efficiency of natural and synthetic communities. Thirty-nine volatile compounds including alcohol, ester, acid, aldehyde and ketone were identified in rice wine mash by HS-SPME-GCMS (Table 2). The use of *L. plantarum* in the brewing process resulted in more production of the following flavour compounds: isoamyl acetate, phenethyl acetate, ethyl octanoate, ethyl lactate, ethyl acetate and isoamyl alcohol. These flavour compounds were considered as important aroma for wine quality since they were recognized as fruity and whisky-liked aroma with lower detection threshold (Baumes et al., 1986). Moreover, acetic acid was lower produced by synthetic community comparing to natural community (**Table 2** and **Figure 1**). Because each rice wine samples contained numerous flavour compounds, for a better visualization and interpretation of the data, principal component analysis (PCA) was performed for identifying correlation and similarity between samples. **Figure 2-A** shows the corresponding factor loading plots establishing the relative importance of each flavour compound found in each sample.

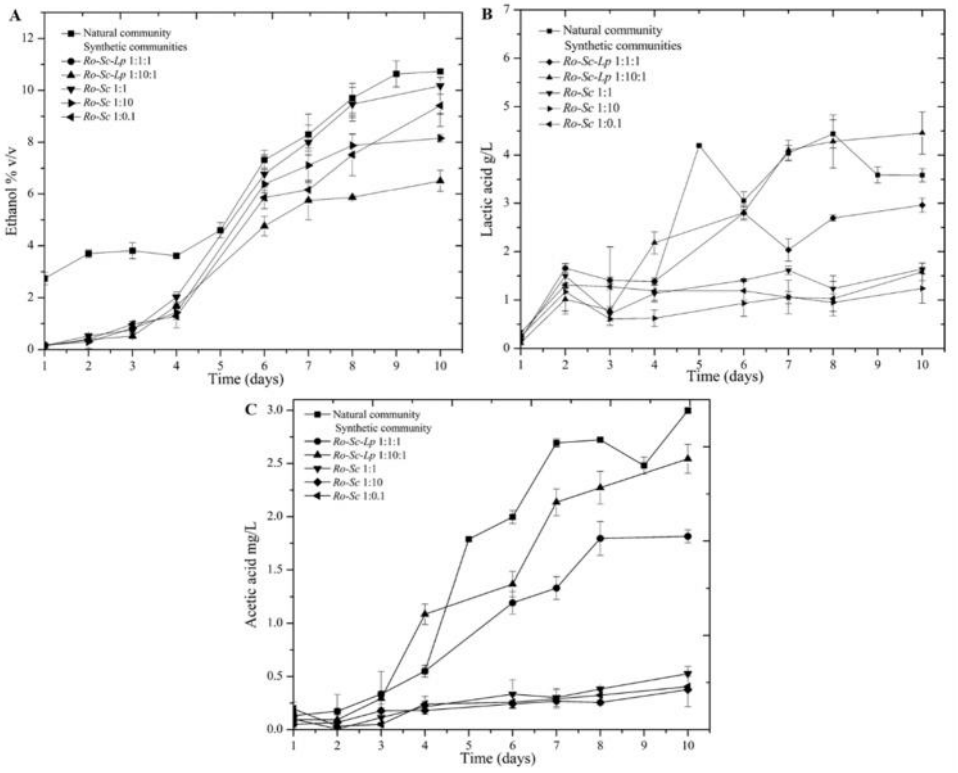

Figure 1. Kinetics of ethanol **(A)**, lactic acid **(B)** and acetic acid **(C)** during fermentation by natural and synthetic communities.

Synthetic communities have been prepared with different ratio between microbial species. In all cases *Rhizopus oryzae* has been inoculated at an initial concentration of 10^6 spores/ml. For the cultures involving either *S. cerevisiae* or *L. plantarum*, their initial concentration is indicated by 0.1, 1 or 10 corresponding to 10^5, 10^6 and 10^7 cells/mL, respectively.

Table 1. Final concentration of ethanol and organic acids at day 10 the end of traditional rice wine brewing process.

Concentration	Natural community	*Ro-Sc-Lp* 1:1:1	*Ro-Sc-Lp* 1:10:1	*Ro-Sc* 1:1	*Ro-Sc* 1:10	*Ro-Sc* 1:0.1
Ethanol (% v/v)	10.73 ± 0.07^{a}	9.95 ± 0.02^{b}	6.5 ± 0.41^{d}	10.17 ± 0.32^{b}	7.95 ± 0.13^{c}	8.41 ± 0.80^{c}
Acetic acid (g/L)	2.99 ± 0.02^{a}	1.81 ± 0.06^{c}	2.54 ± 0.13^{b}	0.52 ± 0.02^{d}	0.37 ± 0.01^{d}	0.40 ± 0.18^{d}
Lactic acid (g/L)	3.58 ± 0.13^{a}	2.95 ± 0.14^{b}	4.45 ± 0.43^{a}	1.64 ± 0.01^{c}	1.23 ± 0.30^{c}	1.58 ± 0.20^{c}

The discrimination of rice wine made by synthetic and natural community was shown in **Figure 2B**. Flavour profile and ethanol yield produced by mixture of those three groups of microorganisms in the same ratio (*Ro-Sc-Lp* 1:1:1) were similar to those of natural community. However, aroma profile produced by the group of *Ro-Sc-Lp* 1:10:1 was distinctly different from that of natural community. The presence of *L. plantarum* in synthetic community influenced significantly aromatic profile of rice wine.

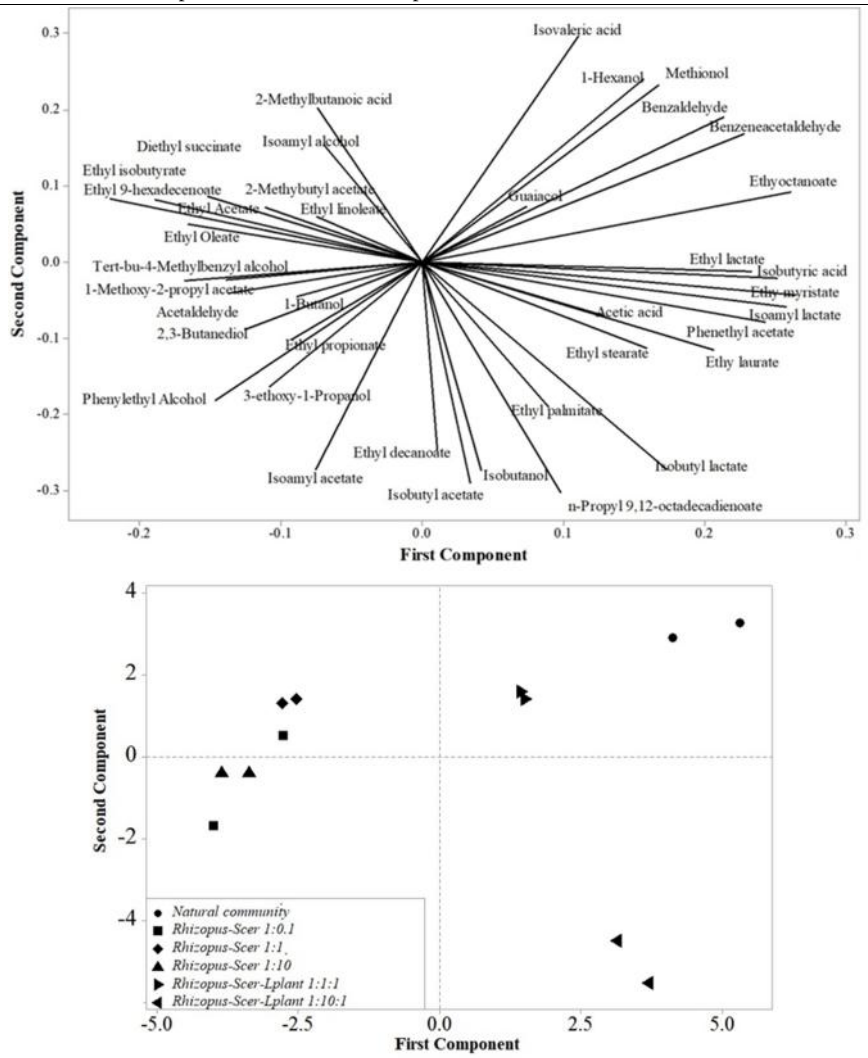

Figure 2: PCA biplot showing aromatic profiles. (A) plot of line distribution of 39 volatile compounds; (B): PCA plot of similarity of each sample.

The same symbols represent the sample from biological replication. Number 1 refer to 10^6 CFU or spores/ml, and 0.1 and 10 refer to 10^5 and 10^7, respectively.

Spatial structuration of synthetic communities based on biofilm cultivation

Impact of submerged and biofilm cultivation on colonization efficiency

Most oenologists are interested in new fermentation technologies for optimizing wine production process, either with quality and/or displaying particular flavour profiles (Viana et al., 2009). However, this kind of study on rice wine production technology is very limited. In the context of mixed cultivation, especially with the presence of filamentous fungi involved in the process, biofilm cultivation design with metal structured packing is interesting in order to understand its impact on biofilm formation and microbial interaction for target flavour compound contributed to rice wine quality. **Figure 3** represents the biomass and microbial evolution on the packing and in planktonic phase during single- and co-cultures.

Based on observation, the maximal biofilm development always occurred at the level of the air/liquid interface. According to **Figure 3-A**, *S. cerevisiae* and *L. plantarum* could grow on artificial rice media containing soluble starch as carbon source. It was also found that in liquid phase, *S. cerevisiae* and *L. plantarum* promoted the growth of each other in a mutualistic way (**Figure 3A, C, D, E**) by comparison with single culture. The presence of *L. plantarum* in all cases did not affect either ethanol production or biomass attached on packing. Ethanol production was observed in significant amount for the cultures made with *S. cerevisiae*, single- or co-culture (**Figure 3B**). When *S. cerevisiae* is absent, only very low amount of ethanol is observed. In the same way, the presence of *S. cerevisiae* in co-culture contributed to higher amount of biomass when biofilm cultivation device was used (**Figure 3C**). On the other hand, there was no evolution neither *S. cerevisiae* nor *L. plantarum* on the metal packing while they were grown without *R. oryzae* (data not shown), showing the role of *R. oryzae* as initial colonization.

Table 2: Flavor compounds (μg/L) produced from natural community and combination of strains as synthetic community in red rice wine brewing process

	Compounds	RI	Natural community		Synthetic community									
					Rhizopus-Sceer 1:0.1		*Rhizopus-Sceer* 1:1		*Rhizopus-Sceer* 1:10		*Rhizopus-Sceer-Plant* 1:1:1		*Rhizopus-Sceer-Lplant* 1:10:1	
			Average	STD	Average	STD	Average	STD	Average	STD	Average	STD	Average	STD
Ester			19506.02		19290.85	1244.58	28584.87	11569.62	33152.54	10743.93	24029.27	6848.21	21738.23	10569.62
1	2-Methybutyl acetate	880	Nd		Nd		55.07	21.45	170.05	4.09	137.89	15.85	Nd	
2	Isoamyl acetate	876	125.98	15.4	344.24	193.83	155.8	9.3	533.14	4.66	270.1	107.15	493.09	123.93
3	1-Methoxy-2-propyl acetate		Nd		18.83		Nd		157.95	33.39	Nd		Nd	
4	Ethyl linoleate		60.44	36.9	Nd		106.44		213.92	195.68	41.39	9.75	Nd	
5	4-Methylbenzyl alcohol		Nd		35.42		30.7		35.16		Nd		Nd	
6	Phenylethyl acetate	1252	1383		98.05	62.35	427.9	371.34	683.62	71.9	1421.55	346.22	1718.85	40.95
7	Ethyl isobutyrate	756	Nd		381.78	175.2	270.53	149.22	296.72	74.47	275.28	61.82	Nd	
8	Ethyl propionate	713	Nd		101.98	10.34	186.71	63.47	416.39	294.43	131.79	55.24	172.86	100.09
9	Ethy myristate	1651	1206.16	63.67	61.84	31.72	80.21	113.44	216.05	56.89	1202.58	70.77	1296.14	123.41
10	Ethyl octanoate	1041	294.15	5.68	20.75		38.33	54.21	41.68	1.137	134.73	18.25	154.17	7.44
11	Isobutyl lactate		51.88	48.02	Nd		8.96	12.68	29.6		43.82	17.34	266.86	14.88
12	Ethyl lactate	1010	4436.35	1501	1137.4		941.16	479.84	2313.94	1345.6	3781.81	1016.62	3941.33	635.91
13	Propyl9,12-octadecadienoate		Nd		20.92	6.88	Nd		Nd		17.65	7.71	59.41	15.7
14	Ethyl stearate		253.6	154.03	20.74		234.15	70.35	179.59	68.49	121.71	37.42	366.97	1.31
15	Ethyl decanoate	1398	63	1.42	90.31	18.23	160.2	106.35	154.6	3.35	65.25	7.81	269.24	204.4
16	Ethyl laurate	1494	281.26	53.27	44.13	18.64	241	144.21	109.99	1.69	213.47	23.88	412.84	1.29
17	Ethyl 9-hexadecenoate		Nd		24.47	12.34	122.79	64.19	61.07	86.37	Nd		Nd	
18	Ethyl Acetate	628	9205.5	1011.45	15713.3	0	20976.76	8697.86	24587.57	7650.87	14189.71	4590.13	4593.61	391.19
19	Ethyl Oleate		189.26	58.42	103.1	53.28	594.82	164.86	669.91	776.57	196.77	86.69	148.4	28.87
20	Ethyl palmitate		1765.79	162.58	537.84	343.77	3420.41	1023.08	1901.87	129.36	1467.66	213.54	7218.18	883.39
21	Isoamyl lactate		149.34	14.15	Nd		Nd		32.67	23.1	34.34	14.56	13423	15.56
22	Diethyl succinate	1167	Nd		12.27		28.61	14.22	22.1	0.78	5.43	1.6	Nd	
23	Isobutyl acetate	776	Nd		57.66	45.7	87.83	2.41	242.27	38.11	214.98	105.78	388.61	13.36
Acids & Aldehyde			7525.45	422.07	588.64		1274.4	209.4	8219.13	5394.32	5551.64	280.47	7215.71	1264.26
24	2-Methylbutanoic acid		254.71	28.66	79.54	82.67	204.84	76.25	352.89	67.68	158.23	56.37	Nd	
25	Isovaleric acid	877	157.19	47.88	22.19	15.69	125.98	15.88	22.24		108.41	18.89	Nd	
26	Acetic acid	600	6050.36	225.56	Nd		85.13	111.22	7576.43	524.84	4602.05	75.1	6487.79	1225.43
27	Isobutyric acid	1215	677.61	82.74	120.04	138.59	420.74		205.59	75.61	536.91	83.6	632.95	16.75
28	Acetaldehyde		Nd		366.87	219.63	387.05		Nd		6.07	5.5	39.12	1.15
29	Benzaldehyde	960	134.63	14.02	Nd		16.61		23.25		40.06	7.93	22.17	14.06
30	Benzeneacetaldehyde		250.92	23.21	Nd		34.05	6.04	38.72	6.18	105.95	38.56	72.78	8.01
Alcohol			46685.3	3990.39	51864.3	27010.77	65315.94	14705.01	68742.36	18432.31	47434.43	1992.35	56613.62	5675.13
31	1-Hexanol	851	108.9	10.82	33.65	47.6	62.15	7.74	14.8	7.34	137.59	4.57	33.29	1.49
32	3-ethoxy-Propanol	833	40.23	2.67	98.85	52.61	29.33	7.05	82.57	12.27	55.19	34.44	64.25	17.715
33	Isobutanol	647	9854.79	262.93	12593.74	10405.16	17018.02		12945.72	2849.94	8041.47	2.28	22432.98	1803.43
34	Methionol	978	266.39	142.01	5.93	8.4	42.58		64.56		266.71	30.07	Nd	
35	Isoamyl alcohol		31276.83	2546.51	24232.3	12570.1	34607.6	11519.76	34191.78	4674.47	30257.95	536.34	21182.44	1471.7
36	Guaiacol	1089	131.53	4.58	49.07		115.24	44.51	125.01	28.39	85.42	13.26	93.52	12.09
37	Phenylethyl Alcohol	1118	3294.85	534.53	6696.66	3835.75	9527.54	2295.83	9903.82	4338.51	3446.39	576.43	8502.17	1331.22
38	1-Butanol	675	167.66	28.22	507.3	143.73	294.54	81.73	895.69		530.19	92.92	343.05	311.04
39	2,3-Butanediol	806	1584.31	460.75	7745.62		3648.25	755.42	10600.96	6533.65	4668.68	736.44	4026.13	744.14
Total			73716.77		71376.9		94788.2		110114.03		77015.35		85567.57	

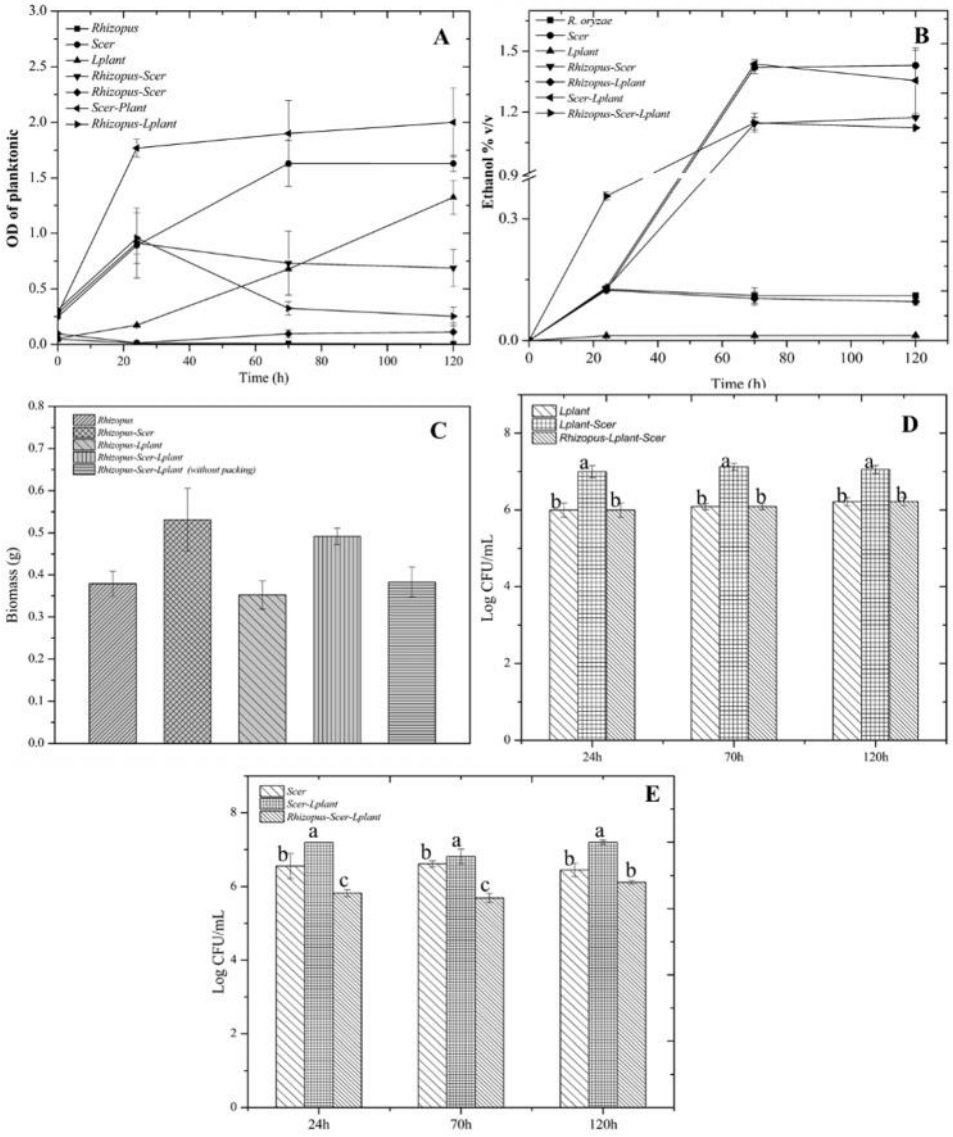

Figure 3. Biofilm and microbial evolution during single and co-culture in biofilm reactor. (A) OD of planktonic phase. (B) Ethanol production. (C) Total biomass of biofilm attached on packing and without packing after 70 h. (D) *L. plantarum* and (E) *S. cerevisiae* plate count while in the planktonic phase. Means with the same letter are not significantly different from each other ($p < 0.05$).

Impact of biofilm mode of cultivation on flavour compound using natural and synthetic communities

Natural and synthetic communities were cultivated in biofilm mode in order to investigate the impact of species structuration on flavour production. As a result, using biofilm cultivation mode led to a significant improvement of flavour compounds production by comparison with submerged culture, for both for natural and synthetic communities (**Table 3**). As expected, the metallic support of the biofilm cultivation device sustained the selective development of *R. oryzae*, promoting the structuration of the whole community. This mode of fungal development is also recognized as increasing secretion abilities, probably leading in our case to a higher amylolytic activity. Additionally, some unpleasant compounds, i.e. pentanoic acid, isovaleric acid and acetic acid, were produced in high amount during classical submerged cultivation and were absent in the biofilm mode of cultivation. This mode of cultivation also led to an increase of some flavour compounds which are important for the organoleptic properties of fermented beverages, i.e. phenylethyl alcohol and isoamyl alcohol.

DISCUSSION

After isolating some microbial strains from a natural community, targeted experiments can be performed for highlighting optimal community compositions that are responsible for the production of specific metabolites through specific biochemical interactions (Lindemann et al., 2016). In this work, we applied this concept for isolating three strains, i.e. *R. oryzae*, *S. cerevisiae* and *L. plantarum*, from a natural community used in Cambodian fermentation starter in order to determine the performances of synthetic community by comparison with the natural one. Indeed, microbial composition has a strong impact on both rice wine quality and yield. Basically, a spontaneous cereal-based fermentation involves complex interkingdom

microbial consortia including fungi, yeasts, lactic acid bacteria (Blandino et al., 2003; Ly et al., 2018).

R. oryzae has been reported as a strong amylase enzyme producer, frequently found in amylolytic ferment starters for rice wine and during saccharification (Dung et al., 2007; X.-C. C. Lv et al., 2015; O'Brien and Wang, 2008; Thanh et al., 2008; G.-F. Xie et al., 2007). *R. oryzae* produces amylase hydrolysing starch into fermentable sugar feeding *S. cerevisiae* and *L. plantarum* (**Figure 4A**). The presence of LAB found in cereal fermentation is important because beside producing lactic acid, LAB is likely to contribute to the production of some flavour compounds and to display some specific metabolic cross-feeding with yeast (Mukisa et al., 2017). The growth of *S. cerevisiae* in fermented food is induced by acidification from bacteria and it adjust its metabolism by secreting a serial metabolite, notably amino acid, allowing the survival of LAB (**Figure 4A**) (Nout and Sarkar, 1999; Ponomarova et al., 2017). The result of this study supports this evidence, both *L. plantarum* and *S. cerevisiae* promoting the growth of each other during the first 24 hours when grown in co-culture. The major flavour compounds found in this study were ester group that provide a pleasant flavour to wine. The type of ester formed depended on the fermentation environment including temperature and level of alcohol (Killian and Ough, 1979). Guitart and his team revealed that high concentrations of amino acids in grape must was also shown to enhance the production of more volatile esters (Guitart et al., 1999). Similarly, red rice is considered as rice containing highest protein as well as free amino acid by comparison with white sticky and non-sticky rice (Zaupa et al., 2015). Beside this, microbial interaction also has a tremendous impact on flavour compound secretion. Based on Table 2, the type and concentration of ester group, as well as other groups, were affected by the combination of species.

Table 3: Flavour compounds (μg/L) produced from natural community and combination of strains as synthetic community in biofilm reactor with artificial rice media.

Compounds	**Natural community**		**Synthetic community** ***R. oryzae/ S. cerevisiae/ L. plantarum***	
	With packing*	**Without packing****	**With packing**	**Without packing**
Isoamyl acetate	1.78±0.3	2.00±0.46	Nd	Nd
Phenethyl acetate	1159.08±69.74	7.92±0.95	Nd	Nd
Ethyl propionate	4.85±0.15	Nd	Nd	Nd
Ethy myristate	3.29±0.1	Nd	Nd	Nd
Ethyl stearate	3.03±0.8	Nd	Nd	Nd
Ethy laurate	Nd	26.58±16.80	Nd	Nd
Ethyl Acetate	3.16±1.19	1.65±0.12	Nd	Nd
Ethyl lactate	Nd	Nd	2.5±0.2	Nd
Ethyl Oleate	9.12±0.98	2.97±1.61	Nd	Nd
Ethyl Isobutyrate	Nd	Nd	16.62±0.89	Nd
Ethyl palmitate	28.33±17.45	3.64±1.07	Nd	Nd
Isobutyl acetate	43.14±6.43	34.01±14.05	Nd	Nd
Isovaleric acid	Nd	23.69±6.19	Nd	275.58±45.67
Pentanoic acid	Nd	Nd	124.72±13.45	593.58±34.23
Acetic acid	23.31±2.63	8.53±6.03	4.13±0.56	1017.38±21.12
1-Hexanol	40.66±6.89	Nd	Nd	18.82±0.93
Isobutanol	3.24±0.3	210.87±140.32	Nd	Nd
Methionol	Nd	4.35±2.75	Nd	Nd
Propanol	Nd	Nd	10.41±2.34	Nd
Butanol	Nd	Nd	10.72±3.23	Nd
1-Methyl-butanol	Nd	Nd	5.04±0.12	Nd
2-methyl-butanol	Nd	Nd	158.59±13.89	Nd
Isobutyl alcohol	Nd	Nd	378.19±16.78	Nd
Isoamyl alcohol	2374.62±64.32	1703.32±19.8	4247.63±234.89	256.69±13.89
Guaiacol	3.29±0.5	Nd	Nd	3594.66±84.91
Phenylethyl Alcohol	1902.86±89.90	0.58±0.41	2017.87±89.78	Nd
2,3-Butanediol	47.80±1.21	Nd	Nd	2070.68±34.37
Benzaldehyde	25.50±5.25	136.39±17.50	91.75±7.62	Nd
Acetaldehyde	2.78±0.67	Nd	Nd	559.68±23.34

Nd: Not detected.

*With packing: metal packing was put in the middle of shake flask.

**Without packing: conventional submerge fermentation flask.

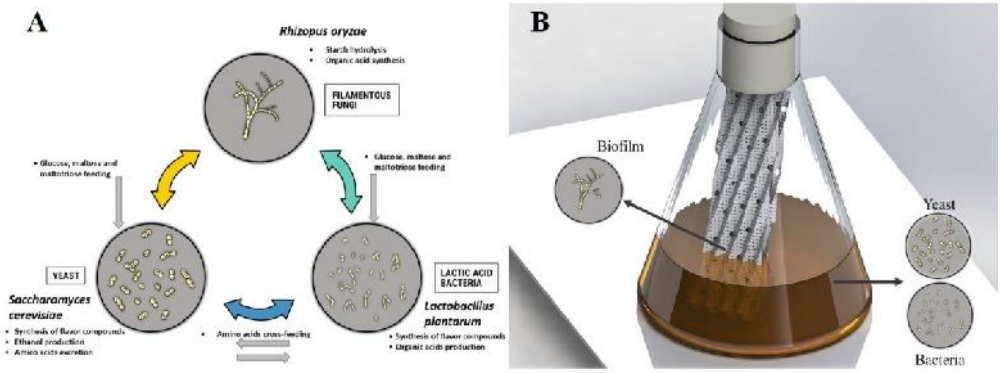

Figure 4. Scheme showing microbial interaction in biofilm cultivation device. **(A)** hypothetical metabolic interactions between fungi, yeast and LAB and the resulting functions during red rice wine fermentation (or on the synthetic liquid medium). **(B)** scheme showing the biofilm cultivation device used in this study, i.e. shake flask with stainless steel packing sheet, as well as the expected position of fungi, yeast and LAB species during fermentation.

Metabolite production can indeed be considerably modified by the microbial composition (Barata et al., 2012; Liu et al., 2017). Based on the results accumulated during this work, we have shown that a simple co-culture *Ro-Sc-Lp* 1:1:1 could provide high ethanol yield and alike flavour profile by comparing to the natural community; moreover, it could reduce some undesired flavours and lower acid which could have off-flavour and taste, especially acetic acid. This might probably because of various and unknown microorganism involved in traditional brewing process and led to difficult control and microbial competitive interaction (Ly et al., 2018). The different combination of these three strains has somehow different impacts on chemical compositions of rice wine including lactic acid, acetic acid, and ethanol yield. These three strains together were able to produce flavour profile which was similar to that from natural microbial community, regularly used in rice wine production in Cambodia.

Beside the optimization of the cultivation medium and consortia members, alternative cultivation device can also be proposed. The selection of microbes for specific aroma profile cannot be effective without understanding how microbe interact with each

other (Liu et al., 2017). In such a case, a biofilm cultivation has been used for promoting the spatial structuration of the three microbial strains extracted from the original starter (Zune et al., 2016, 2015). Indeed, the metallic support used in this device have been previously reported as enhancer the natural binding of filamentous fungi *Trichoderma reesei* (Khalesi et al., 2014) and *Aspergillus oryzae* (Zune et al., 2015). The design of cultivation media promoting metabolic interaction and the design of alternative cultivation device enhancing natural spatial organization of community are both necessary for the study of microbial community (Hays et al., 2015; Klitgord and Segrè, 2010).

Fungal spores generally prefer to adhere and develop on lateral surfaces by several mechanisms involving complex interactions between physical and biological factors. Thus, inserting solid support in the middle flask is very important for the growth of filamentous fungi. Filamentous fungi in submerged and semi-solid conditions always cause problem of increasing viscosity and forming different morphologies leading difficulties of system control during production, notably; cereal-based fermentation (Papagianni, 2004). Rice wine production techniques were commonly performed in simultaneous saccharification and fermentation (SSF) method by which starch enzymatic hydrolysis and alcoholic fermentation occur simultaneously in the same reactor. Using SSF can eliminate the inhibition of saccharifying enzyme by sugar substrate (Rosales et al., 2018) and give unique flavour of rice wine (Dung et al., 2007, 2005). The process of rice wine production operating with enzymatic saccharification and alcoholic fermentation gave distinguish flavour profile. This is why saccharification by fungi, alcoholic fermentation by yeast are still used for rice wine production practice. Recently, novel functionality was achieved within a bacterial–fungal co-culture to create special flavours during fermentation in the food industry (Scherlach et al., 2013). Microbial interactions are very essential for a successful establishment and maintenance of a microbial population (Braga et al., 2016). Synthetic community ecology focuses on designing, building and analysing the

dynamic performance and understanding how community properties appear as a consequence of those interactions (Dolinšek et al., 2016). In the field of biotechnological applications, single- and multi-species culture have a rise of interest in field of research in order to investigate new technology as well as interested metabolites (Liu et al., 2016; Ponomarova and Patil, 2015). The mixture of chemical substance according to rice composition has been used to set up a new technology using corrugated metal in submerge fermentation flask. The support can be considered as an efficient way for bioprocess intensification by promoting the exchanges between the biofilm, gas and liquid phases. The result revealed the importance of metal packing system on the filamentous fungi growth and interaction with other strains during the process. The biomass binding on packing, the growth rate and ethanol production in planktonic were affected by the combination of strains and the presence of solid support. Moreover, the microbial interaction in term of flavour production was really occurred. Even though *R. oryzae, S. cerevisiae* and *L. plantarum* could grow alone in this media, single species was not able to produce some compounds; however, co-culture of three strains could secrete some important compounds such as phenylethyl alcohol, isobutyl alcohol, isoamyl alcohol and 2-methyl-butanol. Additionally, some of off-flavour compounds (pentanoic acid, isovaleric and acetic acid) were reduced in biofilm cultivation mode.

Cambodian people have their own preference for the typical sweet-sour-floral aroma of rice wine. However, keeping or replacing this particular product is a key role of researcher to figure out a synthetic community to produce rice wine in a better control system with similar organoleptic and consistent quality. Consequently, the information from this study can be a part of improvement on Cambodian traditional dried starter and provide an insight understanding on traditional rice wine development. Moreover, the biofilm cultivation device from this study is a tool which enable a step forward in developing and improving the understanding of interkingdom

microbial interactions involving filamentous fungi, yeast and bacteria in the aspect of optimal management of organization of microbial communities.

Author Contributions: SL performed the main experiments and drafted the manuscript. HM and CP accomplished duplicates experiments and reviewed the manuscript. FBK and BF carried out data interpretation and review the manuscript. TK and M-LF performed SPME-GC-MS data analysis. FD designed the experiments and drafted the manuscript.

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Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary Materials:

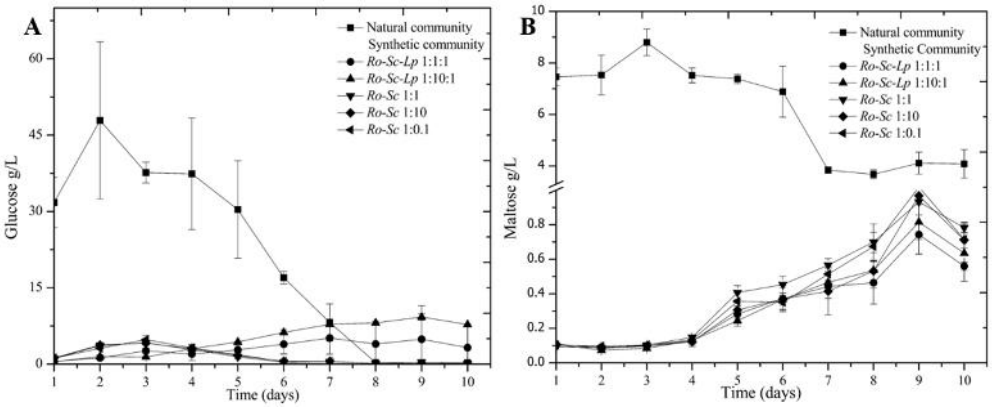

Figure S1. Kinetics of glucose (A) and maltose (B) production during fermentation by natural and synthetic communities. Synthetic communities have been prepared with different ratio between microbial species. In all cases *R. oryzae* (*Ro*) has been inoculated at an initial concentration of 10^6 spores/ml. For the cultures involving either *S. cerevisiae* (*Sc*) or *L. plantarum* (*Lp*), their initial concentration is indicated by 0.1, 1 or 10 corresponding to 10^5, 10^6 and 10^7 cells/mL respectively.

Chapter 3.3: Modulation of fungal biofilm physiology and secondary product formation based on physico-chemical surface properties

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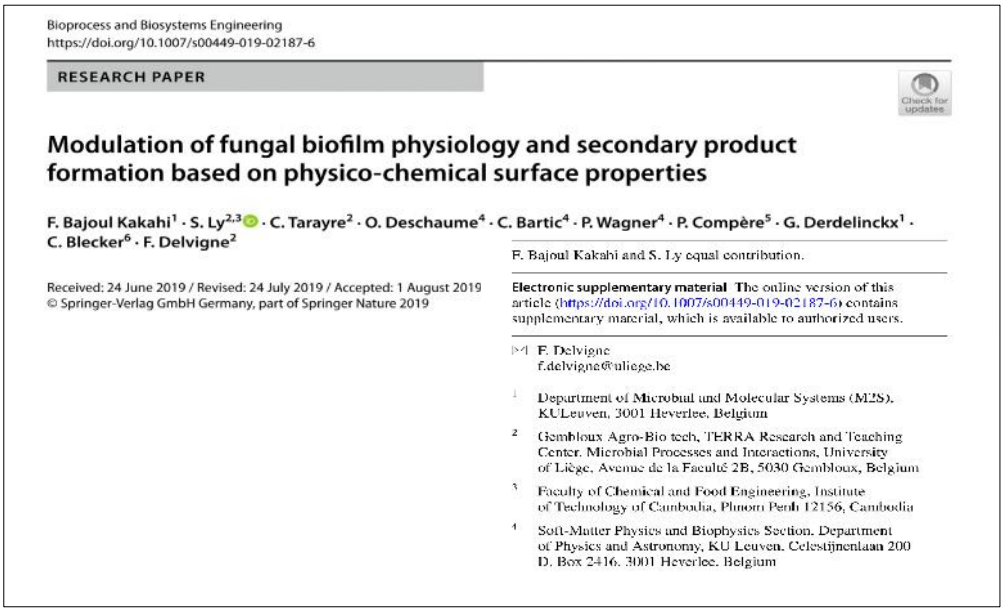

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RESEARCH PAPER

Modulation of fungal biofilm physiology and secondary product formation based on physico-chemical surface properties

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ABSTRACT

Relative to the amount of knowledge concerning bacterial biofilms, little is known about the impact of physico-chemical properties of support material on fungal biofilm adhesion and physiology. In the field of industrial fermentation, large-scale production of low-cost fungal secondary product is a challenging area of research. In the present work, the effect of physico-chemical surface properties of five different materials (Teflon, glass, Viton™ rubber, silicon rubber, and stainless steel) on production of class II hydrophobins (HFBI and HFBII) from *Trichoderma reesei* (HFB2a-2) and *Trichoderma harzianum*) was evaluated. Two culture systems (shake flask and drip flow reactor (DFR)) were used in this study to promote biomass growth and the production of hydrophobins. Furthermore, the effect of physico-chemical surface properties (hydrophobicity, surface energy) and surface texture (roughness) of support material on the initial colonization and attachment of fungal biofilm was evaluated. Maximum biofilm productivity was obtained by using Viton™ rubber for *T. reesei* and Viton™ rubber and stainless steel as support material for *T. harzianum*. Scanning electron microscope (SEM) revealed that fungal biofilm adhesion was higher on the rough hydrophobic Viton rubber surface as compared to the smooth hydrophobic Teflon surface. Initial colonization initiated because of surface irregularities and holes in the material as hyphae filaments. Moreover, compared to traditional submerged fermentation, a significant increase on biofilm productivity for both strains (*T. reesei*, *T. harzianum*) in all five materials was obtained.

KEYWORDS: hydrophobin, solid-state physiology, surface colonization, *Trichoderma*, biofilm reactor.

INTRODUCTION

Filamentous fungi are unique organisms that are generally used for the production of secondary product, such as enzymes or antibiotics. At the industrial scale, several bioreactor designs have been considered for filamentous fungi cultivation, but

submerged fermentation techniques are typically preferred due to their ease of use and their better batch-to-batch reproducibility. However, with this technique, mycelial growth can increase the viscosity of the medium and a high shear rate can affect production yields of the fungi (Fazenda et al., 2008; Ibrahim, 2015). An alternative is solid-state fermentation (SSF), which involves the growth of a fungal biomass on the surface of humidified solid substrates. This system requires less water/energy intake and only needs inexpensive raw materials. Additionally, the development of fungi on such substratum is known to stimulate solid-state physiology leading to improved excretion of products such as pigments, enzymes, and even heterologous proteins when compared with submerged fermentations (Couto and Sanromán, 2006; Marcial et al., 2006) . Nevertheless, large-scale application of SSF is restricted because of oxygen and nutrient gradients inside the solid mass, as well as difficulties with heat removal and downstream processing operations (Bhargav et al., 2008). In a biofilm reactor (BFR), filamentous fungal biomass naturally adheres and colonizes the surface of an inert support in contact with the liquid medium while (Gamarra et al., 2010; Zune et al., 2016) simultaneously maintaining the biological advantages of solid-state physiology. In this work, the impact of fungal biofilm physiology on production of hydrophobin, a valuable secondary product, was tested. Hydrophobins belong to a group of globular proteins secreted by *Ascomycetes* and *Basidiomycetes* (Linder et al., 2005). These proteins help fungal mycelia overcome the surface tension created in air-water interfaces and initiate aerial growth (Wösten, 2001). The molecular weight of hydrophobins ranges between 7 and 10 kDa. Despite their small size, hydrophobins have the ability to self-assemble in elastic monolayers at interfaces, which drastically decreases the surface tension and thus changes the nature of the interface (Paananen et al., 2003; Ritva et al., 2003). These features make them suitable for different applications in several industrial domains such as surface modifications in graphene (Laaksonen et al., 2010), ceramic (Reger, 2011), foaming agent (Cox et al., 2009), biosensors (Zampieri et al., 2010), cell growth (Hou et al., 2009), and fixation of

aroma molecules (Khalesi et al., 2015). Different biofilm reactor configurations are available for promoting the production of secondary product by microbial cells (Coutte et al., 2017) or for microbial biocatalysis (Rosche et al., 2009). However, few studies regarding the impact of physico-chemical surface properties on the formation of biofilm have been performed.

In an attempt to make the screening of secondary product produced by filamentous fungi easier, we considered whether a biofilm cultivation device can serve as a promising tool for the economical production of biomass without the need for expensive harvesting processes. However, a critical element of new fungal biofilm systems is the material used for attachment. This research describes a comprehensive study of the effects of five different materials (Teflon, glass, Viton^{TM} rubber, silicon rubber, stainless steel) on biofilm formation and hydrophobin production by two filamentous fungi, *Trichoderma reesei* and *Trichoderma harzianum.* These materials differ in their physico-chemical surface properties and surface texture, leading to different interactions with the fungi during initial attachment and expansion of the fungal biomass.

MATERIAL AND METHODS

Fungal strains and cultivation medium

Trichoderma reesei MUCL 44908 and *Trichoderma harzianum* IHEM 5437 (kindly provided by the laboratory of Prof. Bruno Cammue at the University of Leuven) were used in the present study. *Trichoderma* minimal medium (TMM) was adapted from (Nakari-Setälä and Penttilä, 1995) with slight changes as follows (g/L): peptone 5, yeast extract 1.75, KH_2PO_4 5, $(NH_4)_2SO_4$ 3.5, $MgSO_4 \cdot 7H_2O$ (0.75), $CaCl_2 \cdot 2H_2O$ 0.75, $CoCl_2 \cdot 6H_2O$ $4.1x10^{-3}$, $MnSO_4 \cdot H_2O$ $3.2x10^{-3}$, $ZnSO_4 \cdot 7H_2O$ $6.9x10^{-3}$, $FeSO_4.7H_2O$ $10.1x10^{-3}$. TMM was supplemented with 40 g of either glucose for hydrophobin (HFBI) and hydrophobin (HFB2a-2) production by *T. reesei* and *T. Harzianum*, respectively, or lactose for hydrophobin (HFBII) production by *T. reesei.*

Preparation of solid support material and cultivation devices

Viton™ rubber (RD128-Vi), stainless steel (RD128-347), Teflon (RD 128-PTFE), and silicone rubber (RD128-Si) were purchased from Biosurface Technology (USA). Microscope glass slides were ordered from VWR International, China. The dimensions of both coupons and microscope glass slides were the same (2.5 x 7.5 x 0.2 cm). All materials were immersed in a solution of 5% (v/v) commercial detergent for 30 min (Azevedo et al., 2006), which were then rinsed in ultrapure water and immersed in 96% (v/v) ethanol for 30 min (Moreira et al., 2015a). The coupons were rinsed again with ultrapure water and autoclaved at 121°C for 20 minutes at a pressure of 1.2 to 1.3 bars before being used in fermentation flasks for the biofilm assays.

Inoculum preparation

Fungal spores were spread on potato dextrose agar followed by incubation for 7 days at 30°C. Spores of *T. reesei* and *T. harzianum* were collected with fresh medium. For each inoculation, a spore suspension was used with a final approximate concentration of 10^5 spores/mL, based on hemocytometer counting.

Shake flask experiment (SFE)

Five different support materials (glass, stainless steel, Teflon, Viton™ rubber, and silicon rubber) were used to investigate the production of three different Class II hydrophobins (HFBI, HFBII, HFB2a-2) (see Fig. S1). Additionally, flasks without support material were considered as a control; i.e., considered to be equivalent to traditional submerged fermentation. Approximately one-third of inert support was immersed in 25 mL of liquid medium in wide-neck 50 ml flasks (VWR). Spore suspension was added to corresponding flasks at a final concentration of 10^5 spores/mL. Conical flasks were then incubated at 30°C in an orbital shaker at 100 rpm for 7 days.

Drip flow reactor (DFR) experiments

Drip flow bioreactors from Biosurface Technology were used in this study. The DFR containing all five different coupons in the chamber was assembled and autoclaved. Prior to inoculation, the DFR was placed on a flat surface at 30°C. Two steps are necessary for this fungal formation: static phase and a continuous phase. In the static phase, each chamber contained a coupon of a given material and was inoculated with a fungal spore solution in which the coupon is completely immerged. Each chamber was then inoculated with 20 mL of spore solution containing 10^5 spores/mL of each fungal strain (i.e., *T. reesei* and *T. harzianum)* separately. The inoculated reactor was then left for 6 hours to permit fungal attachment to the support materials. The effluent tubing was then unclamped and the reactor was placed at a 10° angle. In the final step, the peristaltic pump was turned on to allow the medium to drip slowly (flow rate of 11 mL/hour per chamber) over the coupons. The reactor operated in continuous flow for 40 hours. At the end of fermentation, the dry weight of biomass attached to coupons was determined by the gravimetric method. Two drip flow bioreactors were operated simultaneously and each experiment was repeated as three biological replicates.

Analytical methods

Biomass quantification

Determination of the biomass dry weights in submerged cultures was performed in liquid phase and on solid material supports. After 7 days, liquid phases were filtered through a Whatman No. 4 filter (pore size of 20-25 µm). The support materials were kept out of the bioreactors for 2 h in order to remove excess liquid before further analysis. The biomass dry weight was determined by the gravimetric method after incubation for 24 h at 105°C.

Hydrophobin identification and quantification

Hydrophobin HFBI and HFB2-a2 were extracted as follows: for submerged fermentation, liquid and mycelium were separated by centrifugation (8,500 g for 15 min at 4 °C, Beckman model J2-21). The supernatant was separately collected and the biomass was re-suspended in 170 mM Tris/HCl buffer (pH 9.0) containing 1% SDS at room temperature for a period of 2 hours with periodic shaking. Next, mycelium was centrifuged and the supernatant was recovered and used as a crude extract. Hydrophobin HFBII was extracted as follows: at the end of fermentation, the mycelium was discarded following centrifugation (8,500 g for 15 min at 4 °C, Beckman model J2-21) and the supernatant was used as a crude extract of HFBII. Protein extracts were further purified by chromatography using a reverse phase chromatography RPC column (6.4 × 100 mm; GE Healthcare) at room temperature. Elution was performed as a linear gradient of acetonitrile (ACN) in Milli-Q water containing 0.1% trifluoroacetic acid (TFA) (from 0 to 60%), with a flow rate of 1 mL/min. The subsequent eluate was monitored by UV at 214 nm. Positive fractions containing purified hydrophobins (fractions between 40-50% of acetonitrile) were collected. All collected hydrophobin fractions were confirmed by matrix laser desorption ionization time of flight mass spectrometry (MALDI-TOF) using an Ultraflex II instrument in linear mode with α-cyano-4-hydroxy cinnamic acid as the matrix (Brüker Daltonics, GmbH).

Scanning Electron Microscopy (SEM) analysis

The effect of surface texture on cell attachment under shaking conditions was evaluated by scanning electron microscopy (SEM). Four materials (Viton^{TM} rubber, Teflon, stainless steel, glass) were selected for SEM observation because attachment to Viton^{TM} rubber and stainless steel was good and attachment to Teflon and glass was poor. The 10 x 10 mm samples of supports were collected at the level of the most intense colonization (air-liquid interface). The samples were first fixed by immersion

in 2.5% glutaraldehyde in 0.1M Na-cacodylate buffer at pH 7.4 for 24 hours and were then rinsed three times in 0.2 M buffer solution. The samples were dehydrated by an ethanol series up to 100%, then by critical point drying using CO_2. To view both sides of the supports, the dried samples were mounted on glass slides in a standing position with double-side carbon tape. Detached parts of the biofilms were put aside either right-side up or upside down to view their outer surface and their contact with the substrate. The surfaces of the supports from where the biofilms detached were also examined. All samples were 20 nm that were Pt-coated in a Balzers SCD-030 sputtering unit prior to be observation in an ESEM FEI QUANTA 600 under 15-20 kV accelerating voltage, high vacuum conditions, and using an ET-secondary electron detector.

Physico-chemical properties of solid support material

Sessile drop method

The contact angles of the tested materials (glass, stainless steel, Teflon, Viton™ rubber and silicon rubber) were determined by the sessile drop method using a contact angle meter tensiometer–tracker (OCA 15 Plus, Dataphysics, Germany). These measurements were performed at room temperature (25 ± 2°C) (Table S1).

Atomic Force Microscopy (AFM) analysis

The texture (i.e., roughness) of all five surfaces was analysed by atomic force microscopy in air. Morphological imaging was performed in alternated contact mode using an Agilent 5500 AFM equipped with NCSTR probes (spring constant: 7.4 N/m, resonance frequency: 160 KHz, tip radius of curvature: < 7 nm). Images were treated (i.e., line leveling, roughness calculation) using Gwyddion, a free and open-source SPM (scanning probe microscopy) data visualization and analysis program (Nečas and Klapetek, 2012) .The surface root mean square (RMS) roughness (Rq) was calculated as the root mean square average of the roughness profile ordinates.

Statistical analysis

Turkey's significant difference (HSD) tests were used to compare biofilm attachment to the surface of all five support materials under dynamic conditions. Standard deviations (SDs) for five independent materials are represented by error bars. Differences between groups were considered to be significant when the p-value was less than 0.05.

RESULTS

Solid support in shake flask leads to extra proliferation of fungal biomass relative to classical submerged cultures

Attachment of two different fungal strains (*T. reesei* and *T. harzianum*) to five materials with different characteristics was tested. The materials were selected based on their different physico-chemical surface properties and also their availability for use in process equipment for scaling-up studies. Fig. 1 shows attachment of biofilm of each strain to different inert supports and also biofilm that remained in the liquid phase after 7 days of culture. Since the coupons were partially immersed during shake flask experiments, it was observed that maximal biofilm development occurred at the air/liquid interface. However, biofilm formation on the immersed surfaces was higher for Viton^{TM} rubber, stainless steel, and silicon rubber (Table S2). Fig. 1A shows the amount of biofilm (dried weight) collected from support materials for five biological replicates. For biofilm development by *T. reesei* regarding HFBI and HFBII production, Viton^{TM} rubber and silicon rubber supports showed significantly higher amounts of attached fungal biomass while Teflon had the lowest amount (Table S3).

For HFB2a-2 production of *T. harzianum*, the type of support material significantly influenced biofilm production. The highest amount of biofilm formation occurred with Viton^{TM} rubber, either attached on the surface or floating in the liquid medium. Significantly higher levels of attachment were observed with stainless-steel as compared to the other three support materials (Table S3).

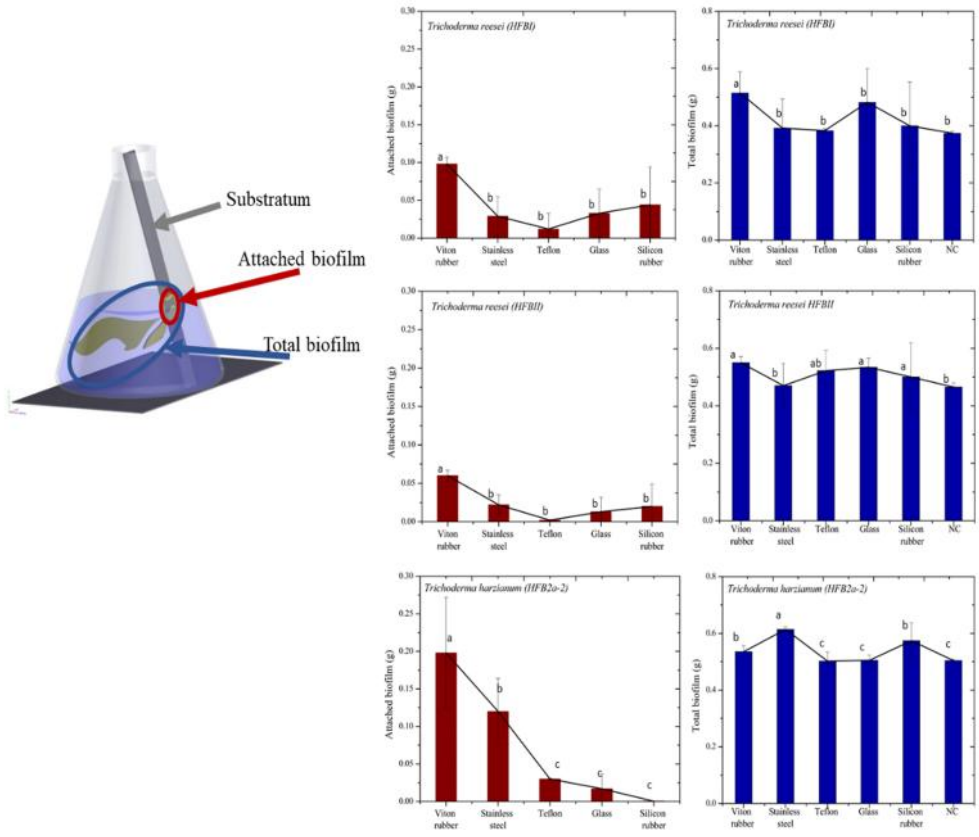

Figure 1: Shake flask experiment. A: Comparison of dry weight (g) attached biofilm on different support materials after7 days of shake flask culture. B: Comparison of total biomass (g) with and without support material in shake flask.

To test the impact of material type on total biofilm production, a flask without a coupon was used as a control. Total biofilm produced by *T. harzianum* or *T. reesei* between five different support materials was observed (Fig. 1B). The results show that insertion of Viton^{TM} rubber, silicon rubber, or stainless-steel coupons into the flask correlated with significantly increased amounts of total biofilm produced by *T. harzianum* as compared to classical submerged fermentation without inert support (Table S4). However, for *T. reesei* (HFBI and HFBII), use of these supports had no significant influence on total biofilm formation as compared to the control (Table S4).

Table 1 shows a comparison of hydrophobin production between traditional submerged fermentation without any support material and fermentation flasks containing five different materials. *T. reesei* exhibited higher biomass production when Viton™ rubber was used as a support system. *T. harzianum* was able to efficiently colonize on stainless steel, Viton™ rubber, and silicon rubber as solid supports. Hydrophobin production by these two strains correlated with the amount of biomass produced. As shown in Table 1, higher production of hydrophobin was observed when stainless steel was used in the system, which resulted in increased biomass production and, consequently, hydrophobin synthesis. Furthermore, the effect of surface texture on cell attachment and morphology of fungi under shake flask conditions was evaluated using scanning electron microscopy (SEM). The selection of the inert support for SEM analysis was based on maximum biofilm production with Viton™ rubber for both *Trichoderma* spp. and minimum biofilm production with Teflon and glass for *T. reesei* and *T. harzianum*, respectively. Surface observations of the different coupons from SEM revealed significant morphological differences between strains, support materials, and carbon sources. These observations not only confirm the impact of surface physico-chemical parameters on macroscopic biofilm physiology, but also higher attachment of hyphae to rough surfaces. For both strains (*T. reesei* and *T. harzianum*), the thickest biofilms were obtained on Viton™ rubber (Fig. 2B, 2D, and 2F), while only a few hyphae filaments attached to Teflon surfaces were observed (Fig. 2A and 2C) and none on glass (Fig. 2E).

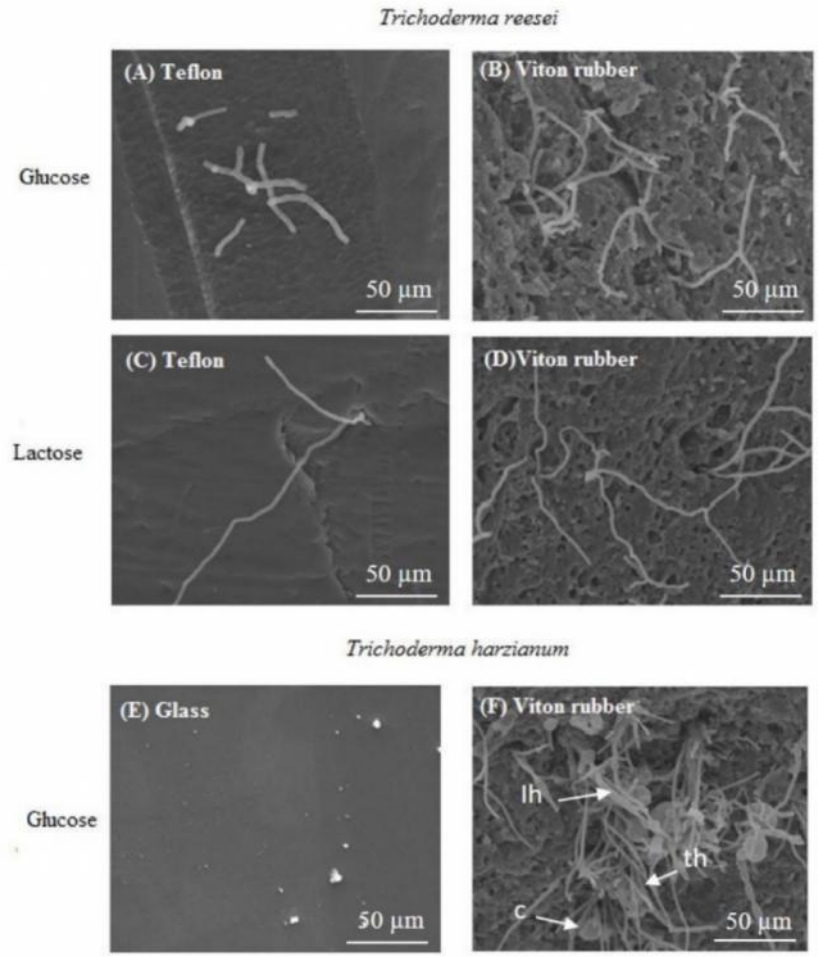

Figure 2: SEM-images of *Trichoderma harzianum* (A,B) and *Trichoderma reesei* (C,D,E,F) biofilms or hyphae on the surface of Viton™ Rubber (B,D, F), Glass (A) and Teflon™ (B and C) after growth with different carbon sources, glucose in case of HFBI production (C) or lactose for HFBII production (E).

A) *T. harzianum* (HFB2a-2) no hypha has been attached on glass. (C, E) Few filaments of *T. ressei* on Teflon showing different branching shapes according to the production of HFBI (C) or HFBII (E). (D, F) Some *T. reesei hyphae* remaining attached to Viton™ rubber after removal of the overlying biofilm; hyphae showed different branching shapes according to HFBI (D) or HFBII (F) production. B) Bunches of hyphae *T. harzianum* (HFB2a-2) hyphae with conidia emerging from holes (arrow heads) of the rubber surface after removal of the overlying biofilm.

The appearance of the Viton™ rubber surface after removal of the biofilms confirmed its roughness and revealed the presence of numerous irregularities, especially small holes that appeared as preferential sites for hyphae anchorage (Fig. 2B, 2D, and 2F). With both strains, hyphae filaments remained attached in these holes and emerged at the rubber surface after removal of the thick biofilm, but for *T. harzianum*, this phenomenon was very evident, with bunches of filaments emerging from Viton™ rubber holes (Fig. 2F). The Teflon surface also contained irregularities that served as settlement sites for hyphae filaments (Figs. 2A and 2C), but these were scarcer in Viton™ rubber, which explains the much lower level of colonization of the surface of this material. A different carbon source (glucose and lactose for production of HFBI (Fig. 2A, 2B) and HFBII (Fig. 2C, 2D), respectively), seem to affect the morphology of the hyphae filament for both strains. Indeed, on Viton™ rubber as well as on Teflon, the filaments of *T. reesei* (HFBI) appeared more ramified with glucose and less ramified with lactose (HFBII) (compare Fig. 2A with 2C and 2B with 2D). Moreover, *T. harzianum* (HFB2a-2) showed different morphology on Viton™ rubber. Thinner, long, and straight filaments of *T. harzianum* (HFB2a-2) at the surface of the biofilm are shown in Fig. 3B while in Fig. 3A, thicker single type filaments of *T. harzianum* (HFB2a-2) can be observed. It also exhibited some thick filaments and fructifications or conidia that are visible through the layer of surface of thin filaments. Theses thin filaments and conidia were also visible when observed from the lower side of the detached biofilm (Fig. 3C).

Optimization of surface colonization with DFR

In the previous section, significant attachment of biofilm was observed. As an alternative, we used the DFR, which is a useful tool that can be used to promote biofilm growth under low shear forces at the air-liquid interface and with a continuous nutrient supply. DFR also allows biofilm to be quantified and to observe its maturation at different culture times and in numerous repetitions (Azeredo et al., 2017). In this

study, DFR was used to investigate the formation of fungal biofilms on five different support materials (Fig. 4 and Table S5).

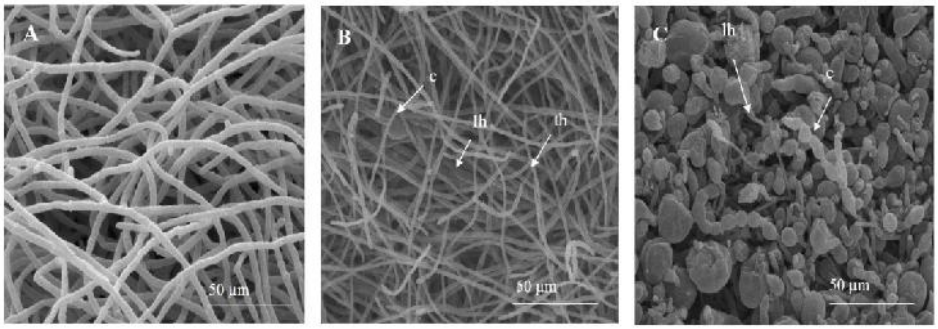

Figure 3: SEM-images of different morphology of *Trichoderma harzianum* on viton™ rubber a) upper surface of the attached biofilm of *T. harzianum* (hfb2a-2) on viton ™ rubber showing a single type of thin hyphae) upper surface of the attached biofilm of *T. Harzianum* (hfb2a-2) on viton[tm] rubber showing thin (th) and thick hyphae (lh) with some fructifications (conidia, c).c) lower side of a detached biofilm of *T. harzianum* (hfb2a-2) on viton™ rubber showing a lot of fructifications (conidia, c) of different shapes.

Table 1: Hydrophobin production yields (mg/L) with different support materials

Hydrophobin	Viton rubber	Stainless steel	Teflon	Glass	Silicon rubber	Traditional submerged fermentation
HFBI *(Trichoderma reesei)*	$45^{a} \pm 2.6$	$34^{b} \pm 2.4$	$35^{b} \pm 3.6$	$36^{b} \pm 3.2$	$37^{b} \pm 1.7$	$35^{b} \pm 2.1$
HFBII *(Trichoderma reesei)*	$34^{a} \pm 3.1$	$28^{a} \pm 1.8$	$32^{a} \pm 4.3$	$33^{a} \pm 2.7$	$31^{a} \pm 3.2$	$30^{a} \pm 1.4$
HFB2a-2 (*Trichoderma harzianum*)	$35^{b} \pm 1.2$	$40^{a} \pm 2.6$	$24^{c} \pm 4.8$	$27^{c} \pm 3.7$	$35^{b} \pm 2.7$	$33^{b} \pm 2.3$

Values in a row Values that are not preceded by the same letter are significantly different ($p \leq 0.05$)

The reactor was maintained for 6 hours; during this time all the spores in contact with the substratum have a better chance to adhere to the surface due to the absence of fluidic movement. After 6 hours, the spent media was removed, theoretically leaving the surface with only adhered spores. Then, the continuous phase started by the

addition of fresh nutritive medium into the inert support with a low flow rate. Spore germination was then followed by a first step of colonization of the whole area provided by the packing, followed by a second step of thickness growth of the fungal biofilm (Harding et al., 2009). The adhered conidia consumed fresh medium that passed continuously through the biofilm. After 40 hours, it was noticed that the entire surface of the coupon was fully covered by the biomass. Dried mass on each coupon was calculated for both strains (*T. reesei* and *T. harzianum)* and compared based on the type of coupon (Table S6). The results in Fig. 4 show that, for *T. reesei (*HFBI and HFBII), Viton^{TM} rubber had the significantly highest biofilm attachment as compared to other materials. Our results showed that, for *T. harzianum,* Viton^{TM} rubber and stainless steel had the largest amount of attached biofilm in comparison with other materials.

A comparative analysis of the biofilm attached on coupons with the different support materials in shake flask experiments and in a drip flow bioreactor is provided in Table 2. Results show that in all cases for both strains, except for T. reesei on silicon rubber, the amount of attached biofilm increased in DFR, which provides a better comparison of the adhesive capabilities of the different materials.

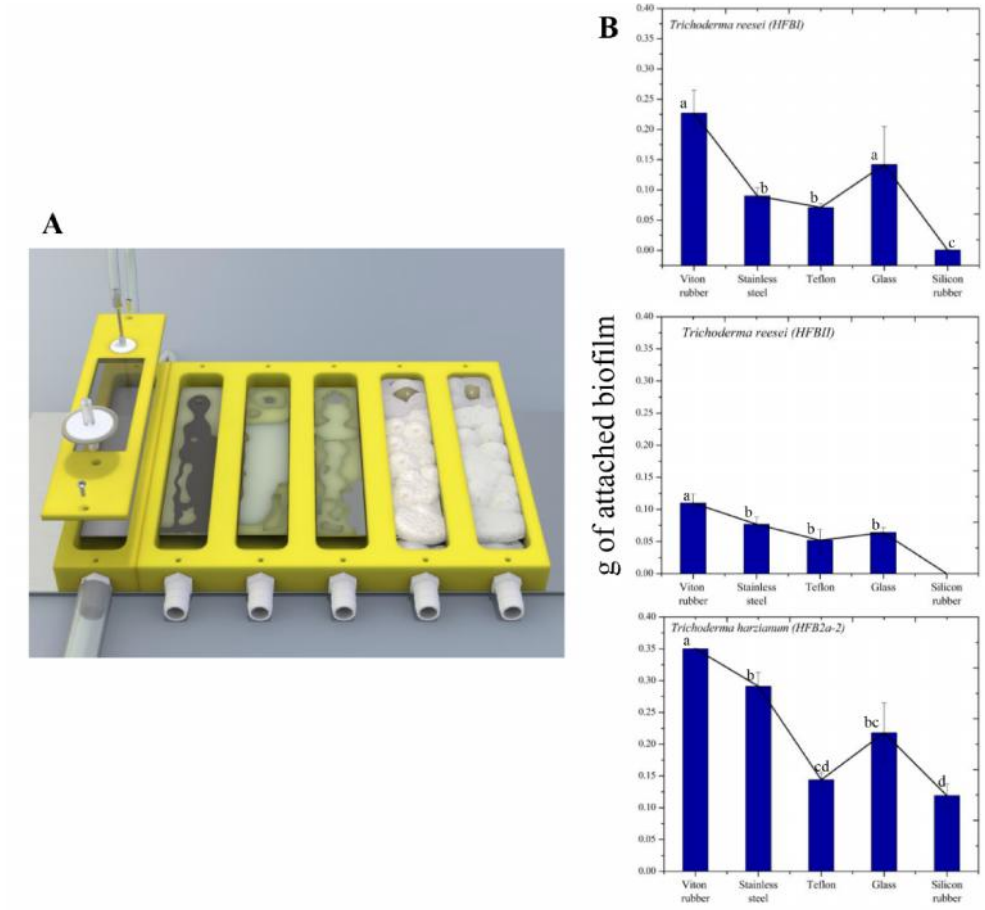

Figure 4: Attachment of *T. reesei*, and *T. harzianum* in Drip Flow reactor. Comparison attachment of biofilm (g) on different support materials after 40h in Drip flow bioreactor. For this formation, two steps are necessary: stationary phase and a continuous phase.

Characterization of the physico-chemical properties of the different support materials and their impact on initial colonization and biofilm formation

Free surface energy, contact angles, and surface roughness properties of the five different surfaces were measured in order to characterize the material surface properties that potentially influence the attachment of fungal filaments. The liquid contact angle varied widely between the five tested substrates (Table S1). Viton™

rubber is considered to be a super-hydrophobic surface (123.5^{o}), while glass served as a super-hydrophilic material (42.6^{o}) compared to the standard 90^{o}. Surface energy calculations were performed by using three liquid contact angles (water-, glycerol-, and dodecane-based), based on the Zisman method. The results showed that Teflon, silicon rubber, and Viton^{TM} rubber have lower surface energies when compared with those of the two other materials. On the other hand, cell attachment and biofilm formation were the highest on Viton^{TM} rubber for both fungal strains (*T. harzianum* and *T. reesei),* as compared to other tested materials.

Surface roughness for all materials was characterized by AFM (Fig. 5). Viton^{TM} rubber had the roughest surface (R_a roughness of 926 nm) while glass was the smoothest, with an R_a value of 11 nm (data not shown). The roughness value of the Viton^{TM} rubber surface was also significantly higher than that of the other tested materials and the roughness value of glass was the lowest (Fig. 5).

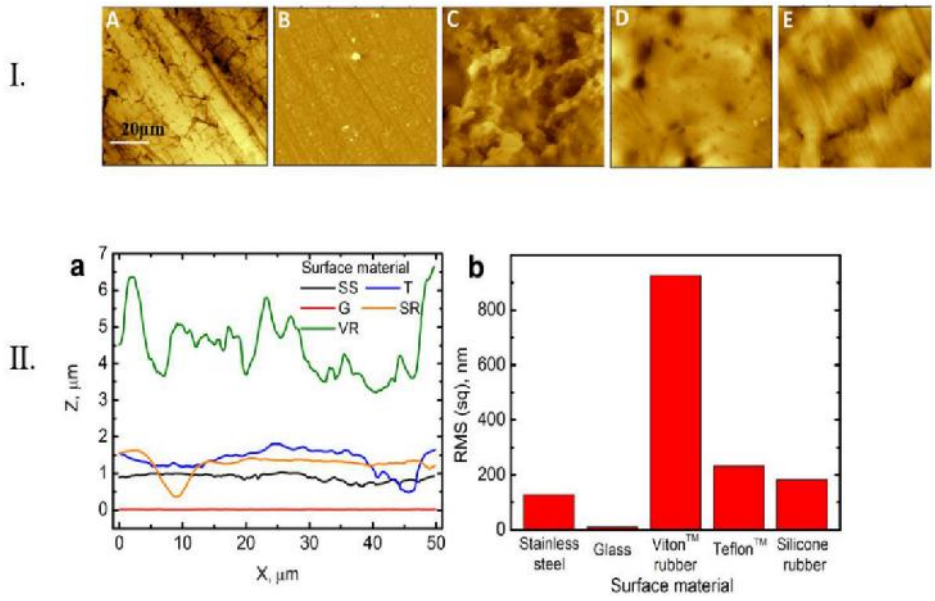

Figure 5: AFM images of the surface roughness of different tested materials; i: A) stainless steel. B) glass. C) viton tm rubber. D) teflon. E) silicon rubber. I: line profiles and roughness values obtained by means of afm for the different materials tested (t=teflon™, g=glass, vr=viton™ rubber, sr=silicone rubber, ss=stainless steel).

DISCUSSION

The production of hydrophobin as a fungal secondary product at an industrial scale with a good production rate and low cost has recently gained the attention of researchers (Khalesi et al., 2014). The design of a specific bioreactor that promotes formation of fungal biofilm on an inert support was used previously in bioreactor testing of the production of hydrophobin HFBII by *T. reesei* (Khalesi et al., 2014) as well as Gla::GFP fusion proteins produced by *Aspergillus oryzae* (Zune et al., 2014). The majority of research that has been conducted on the effect of the surface properties on biofilm formation was focused on bacteria, but almost no reports on filamentous fungal biofilms (FFB) can be found. For this reason, we believe that a new framework for studying this process will be useful.

Fungal spores generally adhere to surfaces by several mechanisms involving complex interactions between physical and biological factors. Physical properties of support, including hydrophobicity, surface roughness, and electrostatic charges are important for the initial attachment step of bacteria, yeast, and filamentous fungi (Webb et al., 1999). There are contradictory results regarding the effect of surface and/or microbial physico-chemical characteristics on microbial attachment (Bos et al., 1999; Chen and Strevett, 2001). Martinez and Casadevall showed that fungal biofilm formation is dependent on support surface characteristics and fungal growth in the biofilm form is less vulnerable to potential environmental stresses (Martinez and Casadevall, 2007). Martinez and Casadevall found that *Cryptococcus neoformans* adhered to all tested materials such as polystyrene, polyvinyl, polycarbonate, and glass but formed the strongest biofilms on a polyvinyl support. For prokaryotes, Gomes and his team revealed the effect of different materials on *E. coli* initial attachment and biofilm formation (Gomes et al., 2015). These authors, as well as many other authors, found a correlation between cell attachment and surface hydrophobicity [36, 38]. Attachment of vegetative cells (Sinde and Carballo, 2000) and bacterial spores (Husmark and Rönner, 1993) is promoted by increased surface hydrophobicity. It has also been shown that other organisms bind preferentially to hydrophobic surfaces; e.g., *Enteromorpha spores* (Callow et al., 2002). Although cell attachment to hydrophobic plastics occurs rapidly, cell attachment to hydrophilic surfaces such as metallic oxides, glass, and metals increases with longer exposure times (Dexter, 1979; Pringle and Fletcher, 1983). Moreira *et al.* revealed maximum bacterial attachment on most hydrophobic surfaces, while, in contrast, the lowest amount of bacteria was detected on the most hydrophilic surfaces (Moreira et al., 2015b). Although the physico-chemical properties affect primary attachment, based on the findings of this study, the relationship between the hydrophobicity of material surface, microbial adhesion, and biofilm formation remains controversial. The impact of these variables on biofilm growth are likely more or less noticeable depending on a combination of

other factors, such as surface texture and cultivation conditions. Indeed, surface texture (roughness) is known to be an important factor promoting biofilm adhesion (Hong et al., 2012). Materials with a suitable surface texture provide a "shelter" for the attached cells. As a result, wash-out of attached cells can be significantly diminished (Gross et al., 2016). Similarly, other studies have shown that an increase in surface roughness promotes bacterial attachment due to the increased contact area between the material surface and bacterial cells (Anselme et al., 2010) and protection of cells from shear forces (Teughels et al., 2006). Roughening of a surface will increase the available area for microbial adhesion. However, if the surface roughness is too high, this may result in instability of the biofilm structure and wash-out of microorganisms (Nakaguma et al., 2003). This is also the case for fungal biofilm, since it has been shown that the attachment of *Aspergillus niger* conidia to a support is moderated by its roughness and adhesive pads (Villena and Gutiérrez-Correa, 2007). During shake flask experiments, shear stress and the limited area of the solid support submerged in cultivation medium induced growth of fungal biomass in the liquid phase. There was an inverse relationship between biofilm quantities attached to the support and biofilm remaining in the liquid phase in all cases. Moreover, the presence of the inert support increased the surface area and induced higher biomass formation together with increased product synthesis. More importantly, as spore hydrophobicity may interfere with spore adhesion, and consequently in biofilm formation, attention must also be paid to cultivation conditions. Jin *et al*. suggested that the carbon source can have an important effect on biofilm maturation (Jin et al., 2004). For instance, different sugars may affect the fungal growth rate and the expression of proteins involved in adhesion or the matrix platform. A *T. reesei* gene encoding a hydrophobin, *hfb1*, was identified as a gene that was highly expressed on glucose-containing medium but not on lactose, xylan, cellobiose, or cellulose (Nakari-Setälä et al., 1996; Nakari-Setälä and Penttilä, 1995). However, *hfb2* is expressed on sorbitol-containing medium, whereas its expression on glucose is relatively low, if

detected at all (Nakari-Setälä et al., 1996). In this study, it was shown that increases in fungal biomass lead to an increase in hydrophobin production. In addition, the excretion capacity is increased when fungal biomass is attached to a given support (Musoni et al., 2015). Therefore, the maximum amount of hydrophobin was obtained when a suitable inert support and carbon source were used in the culture mode. As in the case of *T. reesei* with Viton^{TM} rubber and *T. harzianum* with stainless steel, it was observed as a remarkable illustration that significant increases in total biofilm led to increased production of hydrophobins. Furthermore, SEM-observations revealed that the settlement of fungal biofilm was low or did not occur on the smooth surface of hydrophilic glass. However, it increased on the smooth hydrophobic Teflon surface and reached a maximum on the rough hydrophobic Viton^{TM} rubber surface. According to previous reports, it seems initial colonization started on surface irregularities and holes in the material and hyphae filaments remained attached in these holes, even following mechanical removal of the overlying biofilm (Gharechahi et al., 2012; Lie, 1977; Nyvad and Kilian, 1987). In this report, the filamentous fungi *Trichoderma* spp. were cultivated using two different cultivation devices (SFE and DFR) in order to stimulate hydrophobins production. In DFR conditions, the biofilm formation pattern differed from SFE. In DFR conditions, each chamber contained a coupon of a given material and was inoculated with a spore solution in which the coupon was completely immerged for six hours before drainage, whereas only one-third of the support material was available for spore settlement in shake flask experiments. As expected, fungal biofilm colonization was less visible in flask conditions than in DFR conditions. This is probably because in DFR, low shear stress combined with the continuous mode of operation leads to the favored natural attachment of the fungal biomass.

The results of this study strongly suggest that reduced shear stress and continuous flow of culture media in a drip flow bioreactor significantly increased the attachment of fungal biofilm on almost all support material, except for silicon rubber in the case

of *T. reesei*. As previously reported, initial fungal adhesion can be controlled by surface hydrophobicity, but this process is also strongly dependent on the material and model strain used. More importantly, material roughness directly affects fungal cell attachment and retention and – in some conditions – can help fungal biofilm handle shear stress. This last effect may be more or less visible depending on the hydrodynamic conditions. Our study points out the critical role of natural biological systems that are based on cell attachment and cultivation conditions for improving the production process in terms of the quantity of the final product. This work could be the basis for future research to understand the importance of mixing suitable inert supports, carbon source, and operational mode of cultivation in a bioreactor design in order to achieve optimization and scale-up of secondary product production based on fugal biofilm.

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Conflict of interest. The authors declare no financial support or benefit arising from the research and the authors have no conflict of interest.

Ethical approval. This article does not contain any studies with human participants or animals performed by any of the authors.

Supplementary Materials:

Table S1: Attachment materials and their water contact angel and free surface energy

Attachment materials	**Water contact angle (^{o})**			**Free surface Energy (mJ/ m^{2})**
	Water	**Glycerol**	**Dodecane**	
Glass	42.6	30.75	<2[a]	24.15
Stainless steel	67	70	15	24.12
Teflon	94.6	70.5	28	18.13
Silicon rubber	95.2	69.5	25	19.54
Viton™ Rubber	123.5	93.65	24	20.44

[a] Dodecane contact angles with <2^{o} were below the measurement limit

Table S2: Macroscopic pictures of biofilms attached on different material supports after shake flask incubation

Material	*Trichoderma reesei* (HFBI)	*Trichoderma reesei* (HFBII)	*Trichoderma harzianum* (HFB2a-2)
Viton™ Rubber			
Stainless steel			
Silicon rubber			
Glass			
Teflon			

Values in a row that are not preceded by the same letter are significantly different ($p \leq 0.05$)

Table S3: Comparison of dry weight (g) attached biofilm on different support materials after7 days of shake flask culture

***Trichoderma reesei* (HFBI)**	0.098 ± 0.009^{a}	0.029 ± 0.026^{b}	0.012 ± 0.02^{b}	0.033 ± 0.032^{b}	0.044 ± 0.050^{b}
***Trichoderma reesei* (HFBII)**	0.060 ± 0.007^{a}	0.022 ± 0.013^{a}	0.002 ± 0.001^{b}	0.013 ± 0.019^{b}	0.020 ± 0.029^{b}
***Trichoderma harzianum* (HFB2a-2)**	0.198 ± 0.074^{b}	0.120 ± 0.044^{b}	0.030 ± 0.001^{c}	0.017 ± 0.020^{c}	0.0004 ± 0.000^{c}

Values in a row that are not preceded by the same letter are significantly different ($p \leq 0.05$)

Table S4: Comparison of total biomass (g) with and without support material in shake flask

Strains	Viton™ rubber	Stainless steel	Teflon	Glass	Silicon rubber	Without coupon
Trichoderma reesei HFBI	0.515 ±0.074[a]	0.392 ±0.102[b]	0.383 ± 0.006[b]	0.482±0.118[b]	0.400 ± 0.153[b]	0.374±0.006[b]
Trichoderma reesei HFBII	0.550 ± 0.021[a]	0.470 ± 0.077[b]	0.522 ±0.071[ab]	0.534 ±0.032[a]	0.500 ± 0.119[a]	0.465 ± 0.015[b]
***Trichoderma harzianum* (HFB2a-2)**	0.535 ± 0.021[b]	0.614 ±0.009[a]	0.502 ± 0.032[c]	0.505 ±0.018[c]	0.574 ± 0.064[b]	0.504 ± 0.005[c]

Values in a row that are not preceded by the same letter are significantly different ($p \leq 0.05$)

Table S5: Macroscopic pictures of biofilms attached on different support materials after 40h growth in Drip flow bioreactor

Materials	*Trichoderma reesei* (HFBI)	*Trichoderma reesei* (HFBII)	*Trichoderma harzianum* (HFB2a-2)
Viton™ Rubber			
Stainless steel			
Silicon rubber			
Glass			
Teflon			

Values in a row that are not preceded by the same letter are significantly different ($p \leq 0.05$)

Table S6: Comparison attachment of biofilm (g) on different support materials after 40h in Drip flow bioreactor

Strains	Viton™ Rubber	Stainless steel	Teflon	Glass	Silicon Rubber
***Trichoderma reesei* (HFBI)**	0.227 ± 0.038	0.090 ±0.014^{b}	0.071 ± 0.006^{b}	0.142 ± 0.063^{a}	0.0005 ± 0.000^{c}
***Trichoderma reesei* (HFBI)**	0.227 ± 0.038^{a}	0.090 ±0.014^{b}	0.071 ± 0.006^{b}	0.142 ± 0.063^{a}	0.0005 ±0.000^{c}
***Trichoderma harzianum* (HFB2a-2)**	0.350 ± 0.001^{a}	0.291 ± 0.022^{b}	0.144 ± 0.011^{cd}	0.218 ± 0.047^{bc}	0.119 ± 0.019^{d}

Values in a row that are not preceded by the same letter are significantly different ($p \leq 0.05$)

4

GENERAL CONCLUTION AND PERSPECTIVES

Over 90 percent of the world's rice is produced and consumed in the Asia-Pacific Region. Rice can be transformed to other products to be longer preserved and valorise its value. Among these products, rice alcoholic beverage has played an important role in human spiritual and cultural life in most of Asian country. Rice-based fermented beverage have been known or named as rice wine in most Asian countries such as following: in India (Jeyaram et al., 2008b), in Thailand (Chuenchomrat et al., 2008), in China (Wang et al., 2014), in Korea (Kim et al., 2011), in Vietnam (Dung et al., 2005) and in Cambodia (Chim et al., 2015b; Ly et al., 2018). Beside non-sticky rice, red rice is also used to produce wine (red rice wine), which is particularly desired for its brown-red colour and special fruity aromas. The principle of rice wine production consists of saccharification of steamed starchy source by fungi under solid state fermentation and by yeasts under submerged alcoholic fermentation (Blandino et al., 2003; Dung et al., 2007; Sujaya et al., 2004). In Cambodia, both dried starters and rice wine preparations are made in an uncontrolled environment. Local dried starter and rice wine producers make their product with their own protocol and specific ingredients. This variation might therefore affect the starters' quality in terms of final composition of the microbial consortia found in the starters as well as the final rice wine flavours profile. The organoleptic quality of rice wine is one of the most important characteristics to categorise the grade of wine. The choice of alcoholic ferment starter is one of the most influencing factors on the yield and quality of alcoholic beverage. This current thesis addresses the big concern of rice wine production development in Cambodia. This study showed the first attempt using rRNA pyrosequencing to investigate the microbial composition in Cambodian traditional dried starters and after conduction traditional fermentation. The results from **Chapter 3.1** highlighted the impact of variable pattern structure of microbiota of ferment starters on categories and concentrations of the flavour compounds. The microbial composition of starters varied according to the regions where they were

produced and was influenced by the environment and the material used. Metabolic interactions play an important role in regulating microbial community activities and in maintaining the diversity in microbial communities during the brewing process itself. Although, some species presented in small quantity in the community still have strong correlation in metabolic interaction. The nature of microbial communities in Cambodian traditional starters, their interactions and their contributions to the synthesis of aromas during fermentation is very crucial for rice wine technology development. Most of bacteria species were identified as lactic acid bacteria but they varied in different proportions. Basically, a spontaneous cereal-based fermentation is caused by yeasts, fungi and lactic acid bacteria (Blandino et al., 2003). The genera *Lactobacillus*, *Leuconostoc*, *Weissella* and *Pediococcus* were found on the grains' surface and in the surrounding environment. The interaction between LAB and yeasts has been known to enhance the growth of either group of microbes (Mugula et al., 2003; Omemu et al., 2007) and to build up the alternative flavour production (Mukisa et al., 2017). Ponomarova et al., (2017) revealed that *Saccharomyces cerevisiae* adjust its metabolism by secreting a serial metabolite, notably amino acid, allowing the survival of LAB. Moreover, acid stress can induced the formation of specific aromatic compounds during the lactic acid fermentation (De Angelis et al., 2001; Serrazanetti et al., 2011, 2009). To determine the flavour profile of rice wine mash, SPME method has been optimised and used. SPME has been considered as semi-quantitative method and widely used to determine volatile aromatic compounds in wine (Ha et al., 2014; Jung et al., 2014; Xiao et al., 2014). Flavours compounds were in group esters, alcohols, acids, aldehydes and ketones. The aromas' types and their concentrations might be influenced once more by the substrate composition, the starter culture, the environmental conditions and the process applied. *Weissella, Pediococcus* and *Lactobacillus* genus has most mutually related with flavour compounds. The result from this study, while *L. fermentum* and *L. plantarum* were found as dominant at the end of fermentation in microbial community, the volatile compound was hugely

produced. *Lactobacillus* is an important genus involved in grape fermentation. Recently, some researchers revealed that *L. plantarum* species shows a different enzymatic profile to other LAB species, which could play an important role in the wine aroma profile (Iorizzo et al., 2016; Lerm et al., 2011; Swiegers et al., 2005). Our study has identified dominant strains such as *R. oryzae* as only filamentous fungi, *S. cerevisiae* as ethanol fermenting yeast and *L. plantarum* as important lactic acid bacteria. *R. oryzae* is able to produce volatile compounds during fermentation such as ethanol, 2-methylpropanol and 3- methylbutanol (Bramorski et al., 1998; Christen et al., 2000). Each dried starter contained *Saccharomyces cerevisiae* and *Saccharomycopsis fibuligera.* However, still *S. cerevisiae* was prevalent in the most dried starters and at the end of fermentation. Competitive metabolic interactions among species often play a critical role in the structure and the functions of multispecies communities. The current results here fully improve our understanding of the microbial community exploited in rice wine brewing and the corresponding aromatic profiles. After isolation of consortia members, it is possible to conduct targeted experiments to reveal the possible consortia compositions that are responsible for the production of specific metabolites functions and interactions (Lindemann et al., 2016). These two kingdoms (filamentous fungi, yeast and LAB) were reported to have strong correlation with major flavour compound. Thus, the study of **Chapter 3.2** has been focused on the importance role of these two kingdoms during the bioprocess. The combinations of these three strains were conducted to prior replace the natural community (*Dombea*) made by unknown microorganism and uncontrolled conditions. Therefore, the flavour profile was complimentary to those from natural community (*Dombea*) and some unpleasant flavours were interestingly reduced by using controlled and known synthetic community. This result is very instructive for rice wine production in Cambodia. Thus, an investigation for an alternative bioreactor to deeper understand and better control their interaction has also been studied in this

chapter. Before experimental design of alternative bioreactor of two kingdom started, another part from our study has reveal the importance of biofilm devices for promoting natural binding of filamentous fungal biofilm. The study **Chapter 3.3** tested the appropriate material for the attachment of *Trichoderma* spp. Design of specific bioreactor promoting formation of fungal biofilm on inert support was used previously in bioreactor for testing the production of hydrophobin HFBII by *T. reesei* (Khalesi et al., 2014) as well as Gla::GFP fusion proteins production from *Aspergillus oryzae* (Khalesi et al., 2014). The great majority of researches that have been conducted on the effect of the surface properties on biofilm formation have been focused on bacteria, but almost no reports can be found on filamentous fungal biofilms (FFB). In this study, the filamentous fungus *Trichoderma* spp. has been cultivated using two categories of fermentation systems (SFE and DFR) in order to stimulate biomass binding and hydrophobins production. The study has shown that DFR give better biomass formation on support since early stage of cultivation, this probably due to the low shear stress combined with continuous mode of operation favours natural attachment of the fungal biomass on most of inert surfaces comparing to the shake flask conditions. Physical properties of material surface including hydrophobicity, surface roughness, and electrostatic charge are very important at the initial attachment step of bacteria, yeast, and filamentous fungi (Guillemot et al. 2002 and Webb et al. 1999). Although the physico-chemical properties affect primary attachment; nevertheless, based on this study, the relationship between the hydrophobicity of material surface, microbial adhesion and biofilm formation remains controversial. Even though the impact of this variable on biofilm growth were probably more or less noticeable depending on combination of other factors such as surface texture and cultivation conditions. According to this result, we have also tried to inoculate the filamentous fungi *R. oryzae* isolated from *Dombea* to seek the possibility of new reactor design. As result, the total biomass of *R. oryzae* in dripflow after 40 hours of cultivation reach maximum and had similar amount to total biomass of *Trichoderma*

spp on viton rubber (Data not shown). This means that the natural binding of *Trichoderma* spp. can be used as a model for the study attachment of *R. oryzae*. To study on biofilm formation and interaction, surface energy, surface roughness, chemical properties of the material should be considered to get maximum attachment of biomass. For this reason, we believe that a new framework for studying these processes would be useful. **Chapter 3.2** of our study has used artificial rice liquid media inoculated with interkingdom of three strains *R. oryzae*, *S. cerevisiae* and *L. plantarum* in order to understand the interkingdom interaction for the rice wine process. In submerge culture, fungi regularly exhibit a complex morphological life cycle related to production performance. The complex morphological characteristics of filamentous fungi vary from freely dispersed mycelia to distinct pellets of aggregated biomass. This has been raised as difficulties during the bioprocess. In this work, we have designed a fungal biofilm reactor by using metal packing, generally used in distillation or stripping applications, in middle shake flask. Compared to conventional method, structured packings flask significantly increase capacity and provide high specific surface area, decrease gas pressure drop, enhance gas-liquid transfer rate and display good mechanical resistance (Aferka et al., 2011; Bradtmöller et al., 2015; Olujic and Behrens, 2006; Subramaniyam and Vimala, 2012). In mixed multispecies biofilm, filamentous microorganisms acting as sorbent and source nutrients for the surface attachment of others (Hogan, 2002), this is probably the reason why there were the presence of *S. cerevisiae* and *L. plantarum* while *R. oryzae* grown as thin layer on the packing. The result from this study revealed the importance of metal packing system on the filamentous fungi growth and interaction with other strains during the process providing better control in the system. The results from **Chapter 3.2** and **3.3** shown the importance consideration of biofilm reactor design for filamentous fungi; especially, in the case of microbial interaction study. Biofilm reactors have provided a better control the complex morphological of mycelium.

The instructional information from this study will lead to further consideration for future perspectives. Due to financial constraints, this study focused only one “omic” technology. However, the metabolomic, proteomic and transcriptomic analysis are the interesting and considerable tools to continue deeply understand microbial interaction. In term of the detection coverage, sensitivity and specificity, the results obtained from these microbe-based strongly support the integration of knowledge at different levels in the cascade from genes to proteins and further to metabolic fluxes at a genomic scale is a powerful tool, and will be important for understanding how the individual components in the system interact and influence overall cell metabolism. Biomolecular qPCR should also be further used for studying microbial interaction of this interkingdom. qPCR could provide clear information regarding the targeted DNA sequence which is expressed during the process. Thus, how each strain grow during the process will be discovered. In this case, the study will probably provide a better comprehension of how these three strains work during the rice wine process. These tools together will give deeper interpretation of microbial interaction. More interestingly, rice extract should be used and tested in the biofilm cultivation device to get a deeper understanding of microbial interaction and to move forward in scaling up. Looking forward, rice wine production in an industrial scale should be devoted to develop. This is always challenging for researchers who were successful in the lab scale but met a lot of problems in a bigger scale.

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Appendix –Publication

Publication

1- Modulation of fungal biofilm physiology and secondary product formation based on physico-chemical surface properties

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2- Engineering synthetic microbial communities through selective biofilm cultivation device for the production of fermented beverages

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3- Impact of microbial composition of Cambodian traditional dried starters (*Dombea*) on flavour compound of rice wine: combining amplicon sequencing with HP-SPME-GCMS

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Also published

1- Biochemical Engineering Approaches for Increasing Viability and Functionality of Probiotic Bacteria.

Huu-Thanh Nguyen [1,2], Dieu-Hien Truong [3], Sonagnon Kouhoundé [2], **Sokny Ly** [2], Hary Razafindralambo [4] and Frank Delvigne [2]

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2- Prospects for Food Fermentation in South-East Asia, Topics From the Tropical Fermentation and Biotechnology Network at the End of the AsiFood Erasmus+Project

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