

# Exploring into Multi-Culture Fermentation

By SONG Jianlan (Staff Reporter)

The latitude of 28°N marks a magical line for the brewing/fermentation of wines and liquors. Along this line not only locate Bordeaux, Burgundy and Loire Valleys, but also a “golden triangle” for Chinese liquor fermentation scribed by the most famous three sites of cellars in the country: Renhuai City of Guizhou Province, and Luzhou and Yibin Cities of Sichuan Province. What has made them irreproducible liquor producing areas? The answer could be the combination of favorable climates, water sources and unique microorganisms dependent on the local geographical conditions. With no doubt, the first two elements are both

the endowments of nature, even the last element – the microorganisms alone could be mysterious enough to baffle biologists, though rarely noticed by ordinary people.

The long-lasting puzzle, however, has aroused explorations into the kingdom of microorganisms, and more importantly, driven the recent integration of conventional fermentation with state-of-the-art biotechnologies featuring genomic approaches, high throughput analytic techniques, fermentation kinetics and modeling approaches, producing massive knowledge of modern food technology. As an effort to share and exchange the latest results from such

**Invisible kingdom:** The mud located at the bottom of a liquor cellar of over 300 years old contains a highly complicated, vulnerable microbiota whose composition species are far from clear for scientists. Their resistance to *ex situ* culturing has further increased the difficulty to understand them. And this is just a fraction of the microbiological world involved in fermentation – a biochemical process whose complexity goes beyond ordinary minds.



studies conducted by both the South and the North, and an opportunity for junior scientists from the West and the Oriental to work together, a training course in food biotechnology was launched for developing countries in Luzhou City, Sichuan Province of China from Nov 15 to 19 in 2014, jointly organized by the CAS-TWAS Centre of Excellence for Biotechnology (CoEBio) and the local host, the Luzhou Laojiao Group.

Ensuing the first training course held in December 2013 in Beijing, this term of training focused on food improvement by addressing mixed-culture fermentations, which are widely used in dairy, brewery, alcohol and other food products, under the theme "Unraveling the Complexity of Mixed Cultures in Food Fermentations". The lectures covered a wide range of topics from fundamental theories, mathematical modeling techniques, to recent case studies and site tours.

More than 60 participants from 15 countries, including Bangladesh, India, Iran, Mongolia, Pakistan, Thailand, Yemen, Egypt, Ethiopia, Morocco, Nigeria, South Africa, the Netherlands, Switzerland and China, attended the training.

## Addressing the Challenge

Commonplace as it looks, the mud located at the bottom of an ancient cellar for liquor fermentation could be the habitat of a complicated mixture containing millions of microbial species, which are poorly understood. To date, the efforts for identifying the species have been hindered by their resistance to *ex situ* culturing – according to Prof. LI Yin, head of CoEBio, only less than 1% of the microorganisms in the cellar mud can be cultured in the laboratory. This could make the quality control a nightmare, as the subtle, unknown relationships among the species could evolve towards unpredictable directions during the fermentation and sometimes lead to failure of the culture. Moreover, when multiple cultures are involved, the fermentation process can be even trickier to handle.

Some lectures of the training course provide insights into tackling this challenge from the perspective of fundamental research on the microorganisms present in the mixed cultures, including fundamentals of lactic acid bacteria (LAB), identification and modulation of strains responsible for different properties of the products, reconstitution and optimization of the microbial consortiums via modern genetic/genomic methodologies and mathematical models.

In his three lectures at the opening session, Prof. Jeroen Hugenholtz, a renowned expert in food fermentation,



Prof. Jeroen Hugenholtz gives three lectures under the topic "Functional properties of food & beverages and their improvement using various ingredients".



Prof. Eddy J. Smid from Wageningen University, The Netherlands gives two lectures under the topic "Role and behavior of lactic acid bacteria in complex dairy starter cultures".

introduced recent studies in functional properties of food and beverages, such as flavor, texture, shelf-life, safety and nutritional value. Together with the cases, he illustrated how to "translate" such functionalities into changed strategies for fermentation process and refined ingredients of the starter. He paid special attention to some quite "fashionable" nutrients for healthy foods, like folate and low-calorie sugars, and some antimicrobial products like nisin, which could work as a natural preservative in foods, giving details on how LAB produced them in their "cell factory", and accordingly the development of fermentation starters that could produce such products.

Focusing on the dairy industry, Prof. Eddy J. Smid from Wageningen University, The Netherlands, gave a review on the behavior of LAB in complex dairy starter cultures and the role of nitrogen metabolism in food fermentation processes. When delving into the microbial community structure of complex mesophilic dairy starter cultures, he showed an interesting case in which his research team reconstituted a microbial community containing eight



strains and successfully obtained very good robustness of the ecosystem.

Prof. Jacky L. Snoep from Stellenbosch University, South Africa, gave a course on a fundamental tool for modern food biotechnology: mathematic modeling, and illustrated several cases showing how to model fermentation processes in different cultures.



Prof. Jacky L. Snoep from Stellenbosch University, South Africa and University of Manchester, UK gives a course on mathematical modeling of mixed microbial populations.



A trainee inquires some details involved in the lecture.



Lecturers can not help joining in the discussion in the training.

The contents of the lectures intrigued the participants, sparking intense discussions, inquiries, and inspirations for improving local foods of different continents. Even the lecturers were excited and joined the discussion from time to time.

## For Healthier, Wealthier Communities

Under the spotlight at the training were health-related properties of food/beverages and the technologies to enhance healthy ingredients in the end products through changing the starter compositions and the fermentation processes. Comments by Mr. SHEN Caihong, Vice President and Chief Technology Officer of the Luzhou Laojiao Group might have provided a footnote for this earnest interest in technologies for healthy food at the training.

“The liquor market in China is taking a new trend favoring more economical, more environment-friendly and healthier products, which imposed a great challenge to the liquor industry,” introduced SHEN in his opening address. “To satisfy the new demands,” he continued, “liquor producers need to adopt a more cost-effective, low-carbon mode for production, and furthermore, resort to advanced technologies to increase their yields.”

The lecture by Dr. Floor Hugenholtz, also from Wageningen University, directly answered to such demands. In her lectures she introduced the fundamentals of gut microbiota, its dietary implications, and the latest developments in this field concerning health and diseases, examining it in the relationship network of food, health, and diseases.



Dr. Floor Hugenholtz gives a talk on gut microbiota, a trending topic in the field of biology.



**Yoba for Life:** Dr. Wilbert Sybesma distributing samples of "Yoba" yoghurt to the trainees for a taste, after explaining how the distribution of "Yoba" starters to local communities in Sub-Saharan Africa has worked against poverty and malnutrition. A small sack of starters can sustainably produce homemade "Yoba" of consistent quality for a long time, as long as the users reserve a little of "Yoba" as starter for the next day. The strains in the "Yoba" starter have been optimized according to the local diet, to secure the survival and sustainability of the probiotics they produce in the gut microbiota of locals.

Dr. Wilbert Sybesma from Nestlé, Switzerland, provided a new horizon on how mixed culture fermentations and the related biotechnologies can make a difference in developing countries. He illustrated his ideas with his team's practice of providing a handy, locally fermented beverage containing locally sourced probiotics – "Yoba" yoghurt to local communities in Burkina Faso and Uganda as an effort to mitigate chronic malnutrition and, poverty. Further, he introduced some techniques and natural solutions for strain optimization of LAB, including random mutagenesis, directed evolution, dominant selection schemes and single strain DNA engineering.

## For Modernization of Indigenous Food Industry

Lectures delivered by leaders of traditional Chinese fermentation industry, which revealed the glamour of knowledge in indigenous fermented foods, attracted great attention from participants. The unexpected complexity in traditional Chinese liquor fermentation impressed the participants including experienced food scientists; on the other hand, traditional fermentation techniques and

strategies inherited from this ancient Oriental continent found at the training course a timely breathe of modern methodology, supported by the pouring results from fundamental biology, especially genomic research.

The training course provided a platform for experts and scholars from different countries to meet and learn from each other, especially in the field of modernization of indigenous food industry.

"The conventional solid-fermentation in China, which yields liquor, soy sauce and vinegar, features a multi-bacteria fermentation, of which the reaction mechanism and fermentation dynamics are extremely complex. It urgently calls for analysis by means of modern biotechnology," remarked SHEN Caihong, CTO of Luzhou Laojiao Group with earnest expectation in his opening address of the course. His beliefs were later echoed by Prof. XU Zhenghong from Jiangnan University, Prof. CHEN Gong from Sichuan Academy of Food and Fermentation Industries, and Dr. WANG Songtao, Vice President of Luzhou Laojiao Health Liquor under the Luzhou Laojiao Group, who is also a postdoctoral fellow with the National Engineering Research Center of Solid-State Brewing. Delivering their scientific research results in enriched historic and cultural contexts, all the three lecturers shared the common aspiration of improving the traditional products via modern methods.

Prof. XU Zhenghong gave a brief introduction on brewing of traditional Chinese vinegar (TCV), focusing on their latest efforts to optimize the functioning of microbial community for TCV brewing, aiming at faster fermentation while maintaining its traditional flavors.

Prof. CHEN Gong gave an interesting overview of the latest work of his research team on biodiversity of the LAB consortium for the fermentation of traditional Sichuan Pickles of China. Based on their survey of LAB flora in Sichuan traditional pickles, in which they collected over 180 samples of traditional homemade pickles from over 20 areas in Sichuan and neighboring provinces, they isolated 477 strains of bacteria and yeasts, and identified 17 LAB species belonging to 3 genera from the LAB consortium. Drawing on these results they have set up a microbial culture collection for pickles consisting of 11 genera and 34 species. He further introduced an artificially reconstituted starter (the first direct vat set in China for pickle fermentation) developed by his team containing four strains of dominant LAB from the microbial consortium: "This tiny sachet, which is now available on the market, can produce tasty home-made pickles in just several days or even a few hours, depending on the environmental conditions," he claimed. "Our product still needs further improvement," he





"An old pickle jar could make a valuable trousseau or a good wedding gift in the past," Prof. CHEN Gong started his lecture introducing the enriched, intriguing history of traditional pickle making of Sichuan, China, emphasizing its role in highlighting the bride's housecraft skills. The production of traditional Sichuan pickles could be dated back to very early periods: Even pickle jars unearthed from sites of as early as West Han Dynasty (202 B.C. ~ 8 A.D.) have well-developed shapes and functions, with water seals around the lids to secure good fermentation.



Prof. CHEN Gong talking with a participant from Pakistan after his lecture on traditional Sichuan pickles. The latter wants to integrate the fermentation technology of Sichuan Pickles with that of their local pickles, which are seasoned rather than fermented.

told the author with sparkling aspiration: "Our counterparts in the Netherlands are doing very well: you see, they successfully reconstituted a robust starter containing eight strains. We still have much room to improve. For now, a new generation of first direct vat sets containing six strains is on the way."

Dr. WANG Songtao's lecture covered the multi-culture fermentation process of Luzhou flavor liquor, including the brewing of *Daqu*, the yeast unique for Chinese traditional liquor brewing. This aroused great curiosity among the audiences, some trainees immediately consulted further information about its brewing. WANG also shared details of his research on the bacterial and fungi community dynamics



Dr. WANG Songtao talks about the multi-culture fermentation process of Luzhou flavor liquor.

during the fermentation of *Daqu*, as well as that of the fermenting grains, with dynamic analysis of their physical and biochemical characteristics. Even some experienced researchers were impressed. "We never expected that it could be so complicated to brew a liquor," comments Dr. YU Bo, Vice Director of CoEBio, whose research interests mainly focus on fermentation in extreme conditions. What particularly interested the participants was also WANG's research on the microbial ecology of the cellar mud, a long-lasting mystery as mentioned earlier. In his lecture, he showed some results from his studies on the eubacterial and archaeal community characteristics in cellars of different ages. "Due to the low level of biodiversity, the microbiota in the mud of a new cellar could be instable and probably could fail to produce good liquor," he revealed: "and it generally takes at least 30 years for the microbiota to grow mature," he added.

When asked about the purpose of his studies into the mud microbiota, WANG explained: "We want to identify which microbial species are responsible for certain flavors and other properties, and how the behavior of these specific microbes impacts the flavor and quality of the product. These are extremely important for liquor production."

## Learning by Doing – The Workshop

The workshop is well-anticipated by many trainees, not only because of the intense and exciting process of exploration, but also because of the opportunity it offers the winner to turn his/her dream true – the winner will receive a six-month fellowship to do research at CoEBio, working on his/her proposal.

As a routine, the trainees are encouraged to submit

their research proposals and have them considered by a jury. At the beginning of the training, the 10 proposals on the shortlist will be presented to the whole audience, from which three will be selected for further evaluation. At this point the trainees will be divided into three groups, each focusing on one topic. At the end of the training, the final winner will be elected by the jury after a further presentation session where the three team leaders report their results.

The trainees submitted very interesting proposals for further studies at the workshop, including development of a controlled liquid culture to grow Oyster mushrooms, fermented beverage made of peanuts for Indians, a functional beverage to restore normal gut microbiome for Mongolians, and optimized strains for fermentation of an indigenous beverage in Nigeria called “Burukutu”.

Apparently it was hard for the jury to select the top 3 proposals out of the 10 shortlisted candidates: eventually the jury selected four rather than three. The team members, highly motivated by the explorations and the teamwork, had great fun from their efforts.

“It’s great fun. We work together to detect and solve the problem,” remarked Nilnate Assavasirijinda, a PhD student from Thailand at the CAS Institute of Microbiology (IMCAS), in which the CoEBio is based. She joined a group to develop a fermented beverage based on peanuts. “We are learning a lot in the process. For example, peanuts do not ferment easily; therefore I suggested that we could add some milk to boost the fermentation. However, from my Indian fellows, I learnt that milk is extremely expensive in India and my suggestion would never take place in practice. I never expected that! It is so interesting,” she said excitedly: “Now we have to work hard to find other ways to solve the problem, but it is fun.”

“It’s very nice that you all worked very well on the initial proposals at the second stage and developed your



On the first day of the training, Rediet Sitotaw presents her idea about applying biotechnology to develop a controlled submerged liquid fermentation for growth of Oyster Mushrooms in Ethiopia. She eventually won the champion for the workshop.

own ideas. Now I can tell you that we thought all the proposals were good and certainly deserved a follow-up;” commented Dr. Wilbert Sybesma, mentor of the workshop at the unveiling and awarding ceremony for the workshop winner. Given the excellent performance of the teams, all the four groups gained feedback and suggestions from the jury in person. “For the three teams that are not chosen as the Number One, I’d like to ask you to join us for lunch, then you can have feedback from us. We have to say, although there could be only ONE number one, there can be three ‘Number-tvos’,” he said with a confirmative smile.

In the end, Rediet Sitotaw, a PhD candidate at the IMCAS, won the fellowship for her proposal to do follow-up research on “Application of biotechnology in liquid spawn production of Oyster Mushrooms in Ethiopia”. According to Dr. Wilbert Sybesma, for the other groups, Dr. Floor Hugenholtz and Prof. Jeroen Hugenholtz, who is an expert of food biotechnology, will also offer consulting services.