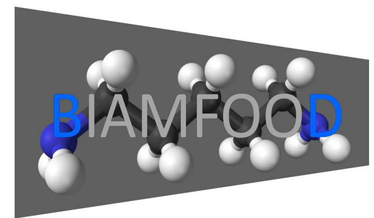

PROJECT FINAL REPORT

Controlling Biogenic Amines in Traditional Food Fermentations in Regional Europe

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Summary

Food fermentations are among the traditional industries that have a long standing history worldwide. In spite of great technological advantages, much of the industry is still based on traditional, experienced-based techniques that are strongly rooted in specific geographic regions. In food fermentations, consortia of microorganisms are essential to the process, and determine the characteristics of the end products. They also are responsible for the production of biogenic amines (BA) that may cause serious human health problems following ingestion of fermented foods containing high concentrations of these compounds. This project focuses on the microorganisms in the food chain that produce biogenic amines with the main goal to improve the quality of traditional fermented food products by reducing/eliminating their biogenic amine content. Control of biogenic amines in the food products will significantly reduce the health risk for the consumer and, thereby, increase the competitiveness of the local industries. The challenge for the food industry is to produce food and beverages in which the levels of BA are minimized. The project focuses on three different fermentation processes in four different region of Europe. The project has both an analytical component aiming at identifying BA producers in the food chain and the conditions that result in BA production, as well as a controlling component aiming at a significant reduction or even elimination of BA in the food products. The whole food chain is considered. At the beginning of the food chain, the potential of microorganisms to form BA is analyzed; during the fermentation process the physiological conditions that result in BA formation are controlled and at the end of the chain the survival of microorganisms producing BA in the digestive tract is analyzed and their effect on the consumer considered.
The distribution of BA-producing bacteria over 1200 bacterial strains for which the genome sequence was available in the public domain and over ~1500 strains obtained from cider, cheese and wine fermentations was determined producing a database of information for studies of biogenic amines in food fermentations. A unique lysine decarboxylase was identified by whole genome sequencing of *Lactobacillus* 30a and a new biosynthetic ornithine decarboxylation pathway was characterized. PCR, HPLC and TLC based tools were developed and optimized in order to detect lactic acid bacteria (LAB) harboring genes involved in the production of the biogenic amines in the food chain. The tools and techniques were used to detect BA-producers and BA in food and in different stages of production of wine, cheese and cider. The physiological role of BA producing pathways was studied in different biological systems demonstrating the relation between acid resistance and turnover through the pathways. Factors affecting the expression of the pathways were determined and a hierarchical catabolite repression regulation of citrate/malate and BA prodicing pathways was demonstrated. BA-degrading isolates from cheese and wine were able to significantly reduce the BA content of the products. Exposure of humanan monocyte-derived dendritic cells and macrophages to BA lead to diminished immunogenic or inflammatory responses. Finally, problems encountered with the expression of transporters of *O. oeni* are not related to the protein family or the host *per se,* but rather protein specific.

Description of the project and objectives

Food fermentations are among the traditional industries that have a long standing history worldwide. Nowadays, companies operating at a European scale are found next to a multitude of more regionally oriented smaller industries producing regional specialties like cheeses, ciders, and wines. In spite of great technological advantages, much of the industry is still based on traditional, experienced-based fermentation processes. In food fermentations, consortia of microorganisms not only are essential to the process, but also determine the characteristics of the end products. Depending on the industrial process, the microorganisms are added to the substrate as starters or are present from nature. In either case, the exact composition of the microbiological flora is not known exactly which may cause adverse effects for the fermentation process, but also for the consumers of the fermented food products. The production of **biogenic amines (BA)** during and following fermentation processes is a well-known example of the latter. The research described in this proposal analyzes aspects of biogenic amine production in foods with the aim to control BA formation. At the beginning of the food chain, the potential of microorganisms to form BA is analyzed; during the fermentation process the physiological conditions resulting in BA formation are controlled; and at the end of the chain the survival of microorganisms producing BA in the digestive tract is analyzed and their effect on the consumer considered.

Biogenic amines are naturally occurring low molecular weight compounds in humans. They are involved in natural biological processes such as synaptic transmission, blood pressure control, allergic response and cellular growth control. On the other hand, biogenic amines may cause human health problems such as headache, palpitations, flushing or vomiting when high concentrations are present, a problem usually caused by the ingestion of fermented foods containing high concentrations of these compounds. High levels of BA in foods may be the consequence of spoilage, but also be inherent to the food production process. **Lactic acid bacteria (LAB)** are the main source of biogenic amines in foods. In bacteria, biogenic amines are the end products of amino acids catabolic pathways. LAB grow on meat, vegetables and fermented foods, which are rich in amino acids that are converted into biogenic amines to produce metabolic energy for the microorganism and to provide resistance against acid stress. The challenge for the food industry is to produce food and beverages in which the levels of BA are minimized.

The **overall goal** of the project is to improve the quality of traditional fermented food products by reducing/eliminating their biogenic amine content. The project focuses on the producers of the BAs in the fermentation process: Lactic Acid Bacteria (LAB). The project has both an analytical component aiming at identifying BA producers in the food chain and the conditions that result in BA production, as well as a controlling component aiming at a significantly reduction or even elimination of BA in the food products. This will be achieved by formulating best practice guides, development of tools, and the utilization of new starter LAB.

The **objectives of the project** are

- to develop and optimize molecular tools for the detection of LAB producing the biogenic amines histamine, tyramine, putrescine, and cadaverine
- to develop and optimize screening methods for the detection of LAB producing BA in cheese, wine, and cider production
- to measure levels of LAB producing BA throughout the fermentation process and identify the conditions under which they produce BA

- to optimize conditions during fermentation to reduce BA formation
- to determine the regional and product specific distribution of LAB producing BA
- to analyze the fate and health risk of LAB producing BA in gastrointestinal tract
- to analyze the impact of BA in the response of human dendritic cells
- to produce starters with reduced potential to produce BA

The project aims at the integration of state-of-the-art technologies and at the implementation of scientific knowledge in the dairy, wine and cider making industries to improve the quality of the products and at the same time reduce the health risk for the consumer. This will be achieved by analyzing the BA producing potential and BA levels and by selecting microorganisms with reduced BA producing potential. The present project is a continuation of the successful project under FP5 entitled 'Fermentation of Food Products: Optimised Lactic Acid Bacteria Strains with Reduced Potential to Accumulate Biogenic Amines' (QLK1-CT-2002-02388) that ended in March 2006. The present project builds upon the previous one, both in the sense of broadening and deepening of essential knowledge, but also will address bottle-necks that showed up in the previous studies. Importantly, in the new project, new insights and technologies will be tested in actual fermentation processes more than was possible in the FP5 project. This was made possible by the availability of new scientific methodologies and by the selection of new project beneficiaries, SMEs and large enterprises, which are producers of fermentation products.

Research in the project is focused on improvement of existing fermentation processes by focussing on the distribution of microorganisms producing BA over different strains, fermentation types, geographical regions, and phylogeny. This will give a complete view on the BA producing potential in food fermentations. The genetic variability is expected to result in optimized tools for the detection of BA producers. Tools for all major BA producing pathways will be developed. The tools will be used to identify the stages in actual fermentation processes where LAB producing BA develop. Further insights in the physiology of the organisms will results in an understanding of why BA are produced during specific industrial practices, which may translate into 'best practice' guides. Currently, no such guidelines exist for food producers at either the national or European level. It is to be expected that, at least in some cases, BA production may be prevented by replacing the biological activity with citrate and/or malate degradation pathways which has the additional advantage that it is generally beneficial to the fermentation process. Alternatively, BA levels will be controlled by inhibitory compounds and/or by LAB with the potential to degrade BA. These studies are expected to result in new starters with the potential to lower BA levels in the final food products. Small scale fermentations will be performed from the beginning to analyze BA producers during the process by existing tools and new tools developed in the project. The project takes the subject one step further in the food chain towards the consumer by an aspect that is new to the field. Rather than studying the adverse effects of ingestion of food containing BA which has been the subject of many studies, the fate of BA producing LAB in food in the human digestive track is studied.

The work plan of the project targets at a number of major questions that are addressed in 6 work packages:

(i) Where do we find BA producing LAB? (work packages 1 and 2)
(ii) Why do BA producers produce BA? (wp 3 and 4)
(iii) If they do, how can we prevent the accumulation of BA in food? (wp 5)
(iv) Do BA producers themselves still form a thread after ingestion? (wp 6)

(i) BA producing pathways are not the main energy and carbon metabolic pathways in lactic acid bacteria and bacteria in general and their presence is not specific to certain organisms or even species. Hence, the capacity to produce BA cannot be linked to a certain species *a priori*. The major goal of **Work package #1** will be to set up a public database containing information on the distribution of BA producing pathways and the related citrate/malate degrading pathways among strains of lactic acid bacteria. Data sources for building the database are twofold. First, the microbial genome sequences available in the public domain that includes ever increasing numbers of lactic acid bacteria and, second, the screening of strain collections from the beneficiaries laboratories for BA producers followed by characterization of the genetic loci. Using data from literature, data obtained in the FP5 project and data obtained from the screens, genes and gene clusters will be identified and, if necessary, linked to the expected biological activity. The database will not only reveal the phylogenetic distribution of the pathways, but also the genetic variability in the coding genes and the distribution over geographical regions and fermentation processes. The database will be used to identify and follow BA producers in actual fermentation processes, which is the major task of **Work package #2**. The information contained in the database will be used to develop new molecular tools for the detection of specific BA producing organisms and to optimize existing tools developed before with respect to the genetic variability according to geographical region and fermentation products. The tools will be used to measure the levels of BA producing LAB in different stages of the fermentation process. The best way to prevent BA production in a fermentation process may be to prevent BA producers from entering the food chain at the beginning of the process. Special attention will be given to the relation between the levels of the organisms and the levels of BA in the final products.

(ii) BA producing pathways are secondary metabolic pathways that become operative in the organisms on demand, i.e. under conditions where there is a special need for them. It is important to identify this 'need' as this may correlate to certain stages in the fermentation process. **Work package #3** focuses on the physiological relevance of BA producing pathways in LAB which is believed to be in metabolic energy production and/or acid stress resistance and on the mechanisms and signals that result in the synthesis of the pathways in the organisms. The challenging objective of **Work package #4** is to replace the BA producing pathways with pathways for citrate and/or malate degradation. The idea is that while the physiological consequences of the two types of pathways, i.e. they respond to the same 'need', would be the same for the organism, the harmful production of BA would be replaced by the beneficial effects associated with citrate and/or malate degradation in the fermentation process. The work mainly focuses on suitable breakdown pathways for citrate and malate, the regulation of expression of the pathways that should follow the same signals and on the existence of a hierarchical regulon in organism in which both types of pathways are endogenously present. In the latter case, it may be sufficient to adjust fermentation conditions to get expression of the 'preferred' pathway.

(iii) In many fermentation processes, the composition of the microbiological flora cannot be controlled at the beginning of the food chain, due to the fact that production practices are non sterile. In fact, a specific, non-starter flora coming with the substrate (milk, grapes, etc) may be essential for the particular fermentation product. **Work package #5** focuses on damage control in those cases where the presence of BA producers in the fermentation process is a fact. Two basically different strategy are followed both aiming at reduced accumulation of BA in the products. One strategy aims at preventing the formation of BA by searching for (food-grade) compounds that inhibit the BA producing pathways. The other strategy allows the formation of the BA by the organism and searches for LAB that, subsequently, are capable of degrading the BA again.

(iv) Clearly, ingestion of fermented foods containing high levels of BA is a direct threat to the human condition. Less visible is the threat formed by the BA producing organism that come with the food and, in principle, introduce the capacity to form BA *in situ* even if the food product itself does not contain high BA levels. The adaptability of food-born BA producers in the human gastrointestinal tract will be investigated in **Work package #6**. One strategy involves the survival rate of the LAB in experimental models mimicking the digestive track. The other strategy involves the study of the interaction of BA producers and human cell lines by analyzing the cytotoxic, antigen-presenting, T cell-stimulatory and polarizing ability of the latter.

In addition, two more work packages were included that have more general purposes. Work package 7 aims at solving a bottleneck in the research of the wine bacterium *Oenococcus oeni* and work package 8 takes the information obtained in the project to the demonstration level.

Work package 7. *Oenococcus oeni* is the most important LAB in the wine industry and responsible for the deacidification of the wine following the alcoholic fermentation by a process termed malolactic fermentation. Previous work has demonstrated that there is a problem with the heterologous expression of (membrane) proteins and genetic manipulation of *O. oeni* in general which will hamper progress in all above work packages. Work package #7 describes a number of approaches to overcome this hurdle which will allow more specific questions to be answered.

Work package #8 describes the technological implementation of applicable results obtained in work packages 1-5. Demonstrations include small scale productions of wine, cider and cheeses involving SMEs and large enterprises that are producers of these fermentation products.

The consortium consists of 10 beneficiaries that are involved in the RTD and Demonstration activities during the whole project (universities, research institutes, SMEs and a major industrial enterprise) and 6 beneficiaries that are involved in the Demonstration activities (large enterprises and SMEs). The mixture of academic and industrial groups aims at bridging scientific fields and spreading excellence to the fermentation industries. Successful completion of the project will contribute to the implementation of the EU policy objectives of improving the competitiveness of European industry and enhancing the quality of life of the European citizen. The participation of 4 SMEs and 2 large enterprises that are producers of fermentation products warrant the immediate implementation of the findings of the project into the fermentations processes when applicable. Biogenic amines are toxic compounds that give symptoms similar to those experienced by food allergy. According to the World Health Organisation (WHO) one of the actions in the the optimal treatment of allergy consists of avoiding the substance that causes the allergy. In line with this recommendation, the BIAMFOOD project aims at minimizing the content of biogenic amines in fermented foods, which undoubtedly is the most effective way of fighting the symptoms. Success of the project will therefore have an important impact on the well-being of a significant part of the European population. The research will help the EC in evaluating whether regulation is required in this area of food safety and regulatory agencies like the European Food Safety Authority (ESFA) to the establish limits on the content of hazardous materials in foods.

Results

Workpackage 1. Phylogenetic distribution of BA-producing pathways in LAB.

The objective of work package 1 was to build a public database of BA-producing pathways and malate/citrate decarboxylation pathways in LAB. The database will contain information on the host organism, coding genes, operon structure, operon context, regulatory elements, genetic element, enzyme classification, transporter classification. Two types of resources were used to obtain the data, available microbial genome sequences in the public domain and strain collections available in the laboratoria of the beneficiaries. Activities of selected ORF's identified as putative genes of the pathways were experimentally characterized to confirm their function. The information in the database will be used to identify and characterize BA-producing LAB in industrial fermentation processes.

BIAMBASE

The BIAMBASE database provides a placeholder for the different databases that were produced in the project. BIAMBASE is published on the web as part of the BIAMFOOD website (www.biamfood.eu). The database contains three sections, PATHWAYS, COLLECTIONS, and EMBEDDING. The first section contains the different pathways for biogenic amine producing pathways and citrate /malate degrading pathways identified in the genomic screen, the second version the BA proding strains identified experimentally in the strains collections of the beneficiaries and, finally, the third section the genetic information on the genetic embedding of the pathways.

BIAMBASE:PATHWAYS. The capacity of microorganisms in general and in particular those involved in food fermentations to produce biogenic amines cannot be linked to a certain species *a priori*. Amino acid and di- and tricarboxylate degrading pathways were analyzed in 1226 sequenced bacterial strains (820 species) and the data was stored in the PATHWAYS database with public access on the internet. The pathways were identified by searching for clusters of a transport protein and metabolic enzymes, i.e. a decarboxylase. The pathways are presented on a fragment of the genome sequence together with the neighbouring genes. Pathways were identified for histidine (hdc), tyrosine (tdc), ornithine (odc), lysine (ldc), citrate and malate. In general, decarboxylation pathways producing histamine, tyramine, and putrescine are found in rare cases in LAB, and the pathway producing cadaverine is not found at all. Ornithine decarboxylating pathways are abundantly found in γ-proteobacteria. An uncharacterized divergently transcribed transporter/decarboxylase pair was found on the genome of a number of lactobacillales. Citrate and malate fermentation pathways were more abundantly found. The citrate pathway in 16 out of 36 *Lactobacillales*. Putative malate fermentation pathways were even more abundant, found in 25 species of *Lactobacillales.* In many cases, the type of pathway correlates with the type of transporters that take up citrate into the cell.

BIAMBASE:COLLECTIONS. The strain collections containing ~1500 strains from wine, cider and cheese fermentations owned by the beneficiaries and mostly isolated from fermentation processes were screened for the presence of BA pathway genes and/or the production of BA. Screens were performed with the techniques developed under work package 2.

Screening for ornithine decarboxylase pathways producing putrescine using genetic probes resulted in very few hits. Nevertheless, putrescine producers were frequently observed in the collections. The putrescine producing capacity was linked to the agmatine deiminase pathway rather than the ornithine decarboxylase pathway. It is concluded that the latter is rare in LAB. About half of a set of *Lactococcus lactis* strains, mostly cheese isolates, were PCR positive for the *agdi* genes but did not produce putrescine. Sequence analysis showed that the genes encoding for the putrescine biosynthetic pathway were interrupted by a 1Kb insertion sequence (IS). Only a single producer of cadaverine was identified (*Lactobacillus* 30a) but the genetic background could not be identified with the available probes. For the other biogenic amines, as a rule, a good correlation between the capacity to produce a biogenic amine and the presence of the genes encoding the decarboxylation pathway was found.
All the *Enterococcus faecalis* strains isolated from cheese or human origin were able to produce tyramine and pustrescine. All the *Enterococcus faecium* strains isolated from cheese or human origin are able to produce tyramine. All the *Enterococcus durans* and *Enterococcus hirae* strains isolated from cheese are able to produce tyramine. All the *Lactobacillus brevis* and *Lactobacillus curvatus* isolated from cheese are able to produce tyramine and putrescine. Some *Lactococcus lactis* strains isolated from cheese are able to produce putrescine.
Cider isolates obtained from both the production areas in Asturia, Spain and Normandie, France were negative for all BA pathways in 84% of the strains. The *agdi* gene was the most frequently encountered BA associated gene (12% of isolates) followed by *tyrdc* (2.5%) and to a lesser extent hdc (~1%) and odc (<1%). Among cider isolates, very few histamine producers were detected.
A collection of 275 wine isolates from the Bordeaux region, France revealed 19 HDC (7 species), 37 TDC (6 species), 4 ODC (3 species), 46 AgDI (10 species) and one LDC-positive strains. The strains were analyzed both for containing the genes as well as for producing the BA. About 15% of the wine isolates from the Apulia region, Italy tested positive for *tyrdc* and *agdi* genes. Several strains of *Lactobacillus hilgardi* and *Lactobacillus brevis* were positive for both *tyrdc* and *agdi* genes. These species are suggested to be the predominant BA-producers in Apulia wines.

Characterization of decarboxylases and transporters

Cadaverine producing pathway in *Lactobacillus* 30a. Among the several hundreds of strains screened, *Lactobacillus* 30a alone was able to decarboxylase both L-ornithine and L-lysine with similar efficiency. The complete genome of *Lactobacillus* 30a (aka *Lactobacillus saerimneri*) was determined to find the genetic background of cadaverine production by this wine bacterium. The genome contained 3 homologues of ornithine decarboxylases (*odc)*, two of which were associated with a transporter. One of the decarboxylase homologues was shown to be specific for lysine. The enzyme is different from the lysine decarboxylases found in Gram-negative and the first LDC detected in LAB. The associated transporter was shown to be both a lysine/cadaverine and ornithine/putrescine exchanger.

Biosynthetic ornithine decarboxylate pathway. A transporter/decarboxylase pair was identified on the genome of a number of LAB, among which *Lactobacillus gasseri* and *Lactobacillus delbruecki,* that are divergently described, normally, in catabolic amino acid decarboxylation pathways, the genes are cotranscribed. The decarboxylase in the pair was annotated as a Arg/Lys/Orn decarboxylase, the transporter belonged to a different subfamily of amino acid transporters than the ornithine/putrescine exchnagers. The decarboxylase and transporter were cloned and functionally characterized. The decarboxylase catalyzed the

decarboxylation of ornithine and diaminobutyric acid (DABA) yielding putrescine and diaminopropane, respectively. The pH optimum of the enzyme was higher than ususally observed for ornithine decarboxylase. The transporter catalyzed uptake of ornithine and putrescine, an activity that was competitively inhibited by the presence of DHAB and lysine. Importantly, the transporter did not catalyze exchange of ornthine and putrescine. It is concluded that the divergently transcribed decarboxylase/transporter pair encode a biosynthetic ornithine/DHAB decarboxylation pathway.

Precursor/product exchangers. The putative ornithine/putrescine exchangers of *O. oeni, Staphylococcus epidermis,* and *Sporolactobacillus sp.* were characterized. PotE of *Escherichia coli* was used as control. The transporters were heterologously expressed in *L. lactis*. It was demonstrated that the clones took up ornithine and putrescine and that accumulated substrates were rapidly chased by external substrates, demonstrating the exchange activity. The histidine/histamine exchangers of *Lactobacillus hilgardii*, *Clostridium perfringens* and *Streptococcus thermophilus* were studied similarly. Accumulated histidine could be chased by histamine demonstrating the histidine/histamine exchange function of the transporters. There was a remarkable difference in affinity for histamine between the transporters from the different origins.

Ornithine decarboxylases. The substrate specificty of ODC's cloned from LAB was determined to see if they could account for cadaverine production from lysine decarboxylation. The ODC from *O. oeni* and *L. brevis* both had a high preference for ornithine, but both had some activity with lysine. The pH optima of the enzymes were 5.1 and 5.6, respectively. The ODC of *O. oeni* BR14/97 was functionally expressed in *E. coli* and purified to homogeneity. The affinity constant Km of the purified enzyme for ornithine was 1 mM and the maximal rate Vmax was 0.57 $U.mg^{-1}$ formed putrescine. Various substrates were tested such as lysine, arginine, citrulline, L-2,4-diaminobutyric acid and α-difluorométhylornithine. No affinity for these substrates could be detected, making the enzyme highly specific for ornithine.

Genetic clustering of BA pathways

Genomic island *S. thermophilus* CHCC1524. The *hdcAPB* cluster of *S. thermophilus* CHCC1524 is located on a genomic insert that is not present in other (sequenced) *S. thermophilus* strains. The complete insert was sequenced and, interestingly, another decarboxylation pathway encoding region was found downstream of the *hdc* cluster on the same insert. The putative pathway contained an aspartate/alanine exchanger homolog and a aspartate decarboxylase. Several elements were identified that possibly are involved in the acquisition of the "genomic island". Upstream, a DNA restriction/modification system (*hsdRM*) and downstream, a partial replicase gene (repA) and a degenerated transposase homolog gene were found. Furthermore, a putative integrase gene and a gene encoding a phage related, putative helicase have been found.

Linking of tyramine and putrescine producing pathways. Analysis of the genomic surrounding regions revealed that tyramine and putrescine pathways genes were always linked on the chromosome of *Lb. curvatus* and *Lb. brevis* while on the genome *E. faecalis* strains these pathways were separated. The putrescine producing pathway involved is the agmatine deiminase pathway.

Workpackage 2. Identifying BA-producers in the food chain

The objective of work package 2 was to extent the set of available molecular tools to detect and quantify the populations of histamine- and tyramine-producing bacteria in cheeses and wines to other products like cider taking into account geographic variability and to develop similar tools for the BA's cadaverine and putrescine. The tools will be used to analyze the presence of BA-producing LAB in different stages of fermentation processes. Strain collections already available in the laboratories of the beneficiaries and new strains identified throughout the project were used as genetic sources for PCR, Q-PCR and Multiplex PCR development.

PCR based tools were developed and optimized in order to analyse lactic acid bacteria (LAB) harboring genes coding for biogenic amines (BA) tyramine (tyrosine decarboxylase gene, *tdc*), histamine (histidine decarboxylase gene, *hdc*), putrescine (via ornithine decarboxylase or agmatine deiminase genes, *odc* and *agd*, respectively) and cadaverine (lysine decarboxylase gene, *ldc*). DNA extraction protocols for detecting BA producing LAB were developed for the different fermented foods analysed (wine, cider and dairy products). Several accurate, reproducible and reilable PCR-based tools (Q-PCR, end-point PCR and multiplex PCR) were developed. Methods were validated on different samples (wine, cider, milk and cheese). Due to different food matrices analysed, methods are sometimes food-specific. However, most of them are usefull for the identification of BA-producers in different fermented food. In addition, a new HPLC method (post-column derivatization) to measure BA was developed and valuated. All the partners involved in the WP striclty cooperate with the cheese, wine and cider producing SME operating in the project.

Q-PCR primers

Q-PCR specific primers for LAB producing histamine, tyramine or putrescine (from agmatine or ornithine) in wine were developed by partner UB2 (Bordeaux, France). New HDC gene sequences of *Pediococcus parvulus, Lactobacillus parabuchneri* and *Lactobacillus sakei* were determined during this project. Primers *tdcf* and *tdcr* were designed from the sequences from *Lactobacillus brevis* (AAN77279), *Lactobacillus curvatus* (BAE02560, BAE02559), *Tetragenococcus halophilus* (BAD93616), *Carnobacterium divergens* (AAQ73505), *Enterococcus faecium* (CAH04395 and EAN10106), *Enterococcus faecalis* (AAM46082 and AAO80459) and *Lactococcus lactis* (CAF33980).

Partner IPLA (Asturias, Spain) designed specific primers for detection of histamine and tyramine producing bacteria in milk and cheese. Four pairs of primers were designed based on the *tdc* gene sequences from public databases and from local strains. One set of primers is useful for general detection of tyramine LAB producers, while the other three sets are specific for *Enterococcus*, *L. brevis* and *L. curvatus*, the main tyramine producers in cheese. One set of primers was developed for general detection of tyramine LAB producers, while three other sets are specific for *Enterococcus* species, *Lb. brevis* and *Lb. curvatus*, the main tyramine producers in cheese. This method was applied to a cheese manufacture, Cabrales, a blue cheese elaborated in Asturias with raw milk, as a model.

The 2 *tyrdc* Q-PCR tools (IPLA and UB2) as well as the *hdc* Q-PCR (IPLA) and *agdi* Q-PCR (UB2) tools were validated on BA-producing cider and wine LAB species as well on various BA-producing bacterial species available in the ADRIA (Normandie, France) and UNFG (Apulia, Italy) collections. For *tyrdc* Q-PCR tools, both methods allowed for the detection, by partner ADRIA, of 7 different species belonging to 4 genera: *Sporolactobacillus* sp., *L.*

brevis, *L. curvatus*, *Enterococcus faecalis*, *E. faecium*, *E. hirae* and *Carnobacterium maltaromaticum* and three different species, by UNFG partner, belong to two genera: *Enterococcus* and *Lactobacillus*. The *hdc* Q-PCR tool allowed for the detection, by ADRIA partner, of 8 different species belonging to 3 genera: *O. oeni*, *L. mali*, *L. parabuchneri*, *L. hilgardii*, *L. sakei*, *L. reuteri*, *Lactobacillus* 30a, *Tetragenococcus muriaticus*.

For *odc* genes, additional nucleotide sequence data for a putrescine-producing *Staphylococcus epidermidis* strain and *L. brevis* strain harbouring the *odc* locus, were provided by partner UB2 and ADRIA, in addition to sequence related to *Lactobacillus* 30a and *O. oeni* strains, previously identified as putrescine producers via the *odc* locus.

UB2 detected *Lactobacillus* 30a (*L. saerimneri*) as cadaverine producer. Indeed, analysis of strain *L.* 30a by TLC (see below) revealed that it produces not only histamine and putrescine, but also high levels of cadaverine when it is grown in the presence of lysine. Following many different approaches, the gene coding for the lysine decarboxylase (LDC) responsible for cadaverine production was identified by whole genome sequencing.

Detection methods

Partner **ADRIA** developed a multiplex PCR method for the simultaneous detection of potential histamine, tyramine and putrescine producers (Figure 1). The multiplex method has since been used by 4 different partners in the project (**ADRIA, UB2, UNFG, IR**) for screening LAB collections for BA producers (wine and cider isolates)

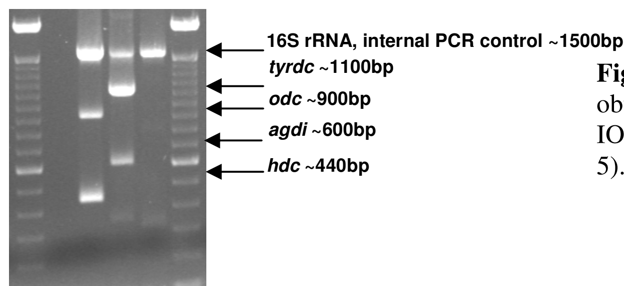


Figure 1. The Multiplex PCR method. Products obtained for *Lactobacillus* **L30a** (lane 3), *Lb. brevis* IOEB **9809** (lane 4) and *O. oeni* ATCC **23277** (lane 5). 100bp DNA ladder (lanes 1&6)

Partner **UB2** developed a new, reliable, cost-effective and rapid method based on thin layer chromatography (TLC) and UV detection in order to identify BA producing strains (Figure 2). Methods was also applied to BA detection in LAB culture supernatants. Methods was also confirmed on BA producing strains collected by partner UNFG.

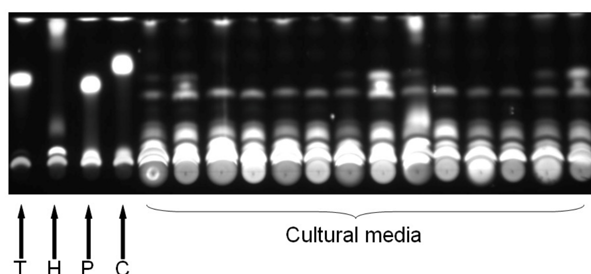


Figure 2. Examples of TLC analysis developed by partner UB2 and applied on LAB culture supernatants. Tyramine (T), Histamine (H), Putrescine (P) and Cadaverine (C).

Partner **IR** developed and validated a reversed-phase separation by HPLC and UV-vis detection of aminoenones formed by the reaction of amino acids, biogenic amines, and ammonium ion with the derivatization reagent diethyl ethoxy ethylene malonate (DEEMM).

Aminoenones formed by the reaction of amino acids, biogenic amines, and the ammonium ion with the derivatization reagent DEEMM are separated by HPLC. Reaction takes place in methanolic alkaline medium for 30 min in an ultrasonic bath. Further heating at 70°C for 2 h produces complete degradation of excess derivatization reagent and byproducts. The reliability of the method was satisfactory in terms of linearity (from 0.5 to 20 $mg.L^{-1}$), precision (relative standard deviation below 5%), recovery (from 98.7 to 101.1%), and sensitivity (detection limit below 0.1 $mg\ L^{-1}$). The proposed analytical method has the following advantages: easy derivatization of wines, quantification of biogenic amines (Histamine, Methylamine, Ethylamine, Tyramine, Putrescine, Cadaverine, Phenethylamine, and Isoamylamine), and complete degradation of excess derivatization reagent during sample preparation to preserve the column.
The method has been tested by other partners (UB, IPLA and ADRIA) and is available on the BIAMFOOD website. Validation following an alternative oenological analysis method for the validation, quality control and uncertainty assessment (IOV Oeno 10/2005 http://www.oiv.int/) was done for 8 biogenic amines (histamine, methylamine, ethylamine, tyramine, putrescine, cadaverine, phenethylamine, isoamylamine). Regular comparison networks to compare results obtained were done with success. This method will be proposed as a reference method to the International Organization of Vine and Wine. Q-PCR based tools and the HPLC methods developed, were furthermore used to detect BA producing LAB during the fermentation processes of wine, cider and cheese and in the final products.

BA content during fermentations and in foods
Randomly purchased commercial cider samples from the Northwest of France (Bretagne and Normandie regions) and samples collected during the elaboration process of ciders from France were analyzed for their BA-content (HPLC) and for the presence of BA-producing bacterial strains (qPCR). Overall, BA levels in commercial ciders were quite low and only some minor differences in amine contents were observed. Samples from the elaboration steps of cider were analyzed by HPLC and corresponded to the beginning and end of the MLF. For these samples, histamine, tyramine, putrescine and cadaverine were all detected with a maximum of 5 mg/l and highest average amine concentration value corresponding to putrescine (Table 1). In addition to putrescine, histamine and cadaverine were detected in the samples that corresponded to the beginning of the malo-lactic transformation (~1% complete) while at the end of the MLF (>99% complete) tyramine appeared.

Sample	MLF	Histamine		Tyramine		Putrescine			Cadaverine	Total
		HPLC	RTQ-PCR	HPLC	RTQ-PCR	HPLC	RTQ-PCR	RTQ-PCR	HPLC	HPLC
							agdl	odc		
VDV-078	1%	2	34,07		nd	3	32.1 (Lb)	20,16	2	7
VDV-137	1%	2	Nd		nd	3	30.36 (Lb)	29,78	2	7
VDV-140	1%	2	nd		nd	4	31.13 (Lb)	nd	2	8
VDV-141	1%	2	32,07		nd	4	31.21 (Lb)	29,6	3	9
VDV-142	1%	1	nd		nd	4	29.5 (Lb)	29,35	3	8
VDV-17Z	100%		35,68	1	29,4		21,16	20,98		4
VDV-29Z	93%		nd		29,6	5	26,37	25,17		5
VDV-4Z	99%		34,7	1	29	4	19,18	21,35		5
VDV-146		1	nd		31		33,97	31,05	1	2
average		**1.62 ± 0.52**		**1.0 ± 0**		**3.86 ± 0.69**			**2.17 ± 0.75**	**6.11 ± 2.26**
max		**2,00**		**1,00**		**5,00**			**3,00**	**9,00**

Table 1. Average and total BA content determined by HPLC analysis for cider samples during the elaboration process. Real-time qPCR results are also given.

Two types of ciders elaborated in Asturias, one elaborated with apples from different origins of Spain and France, and the other one made with apples from Asturias were analyzed in different steps of elaboration. Histamine and tyramime were not present in any of the

analyzed samples. Putrescine was the only BA detected with values ranging from 2.37 mg/l to 4.88 mg/l.
Wine samples collected during malolactic fermentation, storage or commercialy sold, from the Bordeaux, Bourgogne, Rhone Valley and Apulian regions were analyzed for their BA-content and for the presence and/or persistence of BA-producing bacterial strains. Overall, BA levels in wines were quite higher for putrescine and histamine. In contrast, low levels of cadaverine and tyramine were detected. In Figure 3 examples of average BA contents in wine samples in analysed Bordeaux, Bourgogne, Rhone Valley and Apulian regions is shown.

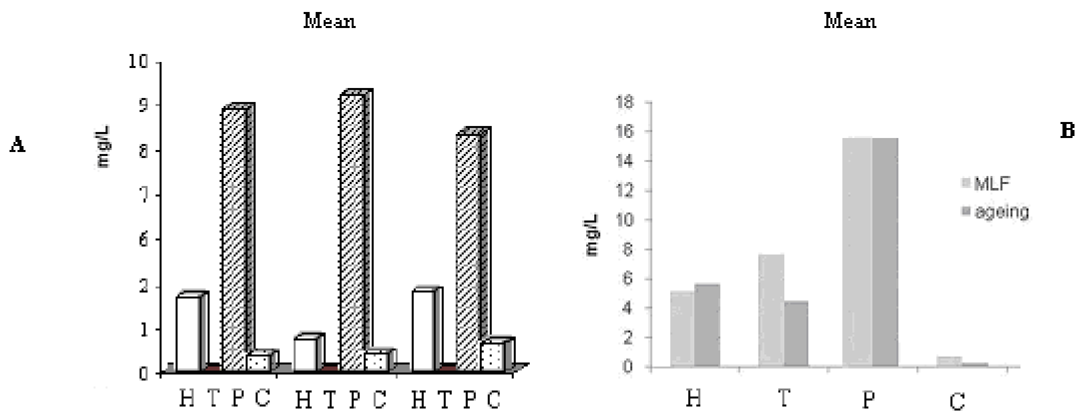


Figure 3. BA content in red wines produced in Rhone Valley and Apulian region.
Red wines commercialy sold or collected during malolactic fermentation **(A** and **B)** or after storage (**B**) were analysed by reverse phase HPLC for BA quantification. Histamine (H), Tyramine (T), Putrescine (P) and Cadaverine (C). Graph shows mean values of three independent measurements.

As reported, putrescine was always identified with concentration ranging from 2 mg l^{-1} to almost 17 mg l^{-1}. In contrast histamine was identified in some samples and ranging from 4 and 8 mg l^{-1}. Tyramine was below the detection limits in Apulia wines, while concentration between 4 to 2 mg l^{-1} were reported for wines analysed by partner IR.
Randomly purchased cheeses were analysed for the presence of histamine by HPLC analysis. Histamine was detected in 41.25 % of the samples with values ranging from 20 to over 1000 mg Kg^{-1} (mg of histamine per Kg of cheese). The highest histamine content was detected in blue cheese samples (1041 mg Kg^{-1}). The highest histamine concentrations were observed in long ripening cheeses. Histamine concentration was also determined in processed cheeses (entire, portions, grated and sliced).

Correlation between the type and persistence of the identified BA-producing LAB and the amounts of BA in wine and cider was always confirmed. Several LAB harbouring genes coding for agmatine deiminase and ornithine decarboxylase (both involved in putrescine production) and histidine decarboxylase (involved in histamine production) were identified in foods. LAB strains able to produce BA (analysed by HPLC or TLC) mainly corresponded to *O. oeni, P. parvulus, L. plantarum, L. hilgardii, Lb. curvatus*, *Lb. brevis* and *E. faecalis*. Occxasionally putrescine and tyramine clusters were found to be linked on the chromosome (e.g., *L. curvatus* and *L. brevis*), but in other cases they were separated (e.g., *E. faecalis*). In cheese, where tyramine and histamine are the main BA detected, histamine and tyramine producing bacteria were detected using different tools including Q-PCR. *Enterococcus* sp. were identified as the main tyramine producing bacteria.

Workpackage 3. Physiology of BA-producing pathways: Metabolic energy production and acid resistance.

Different BA-producing LAB have to cope with different and variable food environments characterized by very different constraints, such as those set by the levels of available substrates or pH values. It is believed that LAB benefit from BA-producing pathways because of the production of metabolic energy and/or better survival in acidic environments. The objective is to evaluate the exact role of the pathways in different species/strains and under different conditions. The physiological role of HDC, ODC, LDC, TDC and AGDI pathways was studied with strains harboring BA-producing capacities on endogenous plasmids using cured strains as controls, with BA-producing and naturally non-producing isogenic LAB as controls or with a recombinant *L. lactis* strain carrying a plasmid coding for the histidine decarboxylase pathway of *Streptococcus thermophilus*. The strains were isolated from fermented products (wine, cheese and cider) or from the gastrointestinal tract (*Lactobacillus sp*. 30A). The relation between available N sources and the capacity to produce BA was studied and the factors influences the induction of the pathways determined.

Physiology of BA-producing LAB from fermentations

Histidine decarboxylation (HDC) pathway. The wine strains *Lactobacillus hilgardii* IOEB 0006 (HDC+) and *Oenococcus oeni* IOEB 0607 (HDC+) and their plasmid cured (HDC-) isogenic derivatives. showed intrinsic resistance to extremely acidic environment (pH<2.0). The presence of the HDC pathway strengthened this resistance in *L. hilgardii*, whereas no difference was detected in HDC+ and HDC- *O. oeni* strains. This suggests that acid tolerant wine bacteria can use the HDC pathway for growth rather than for acid resistance.
In *Lactobacillus reuteri,* the data obtained for strains DSM 20016 (HDC+) and NS98 (HDC-) suggests that the HDC pathway impacts both acid-resistance and growth of HDC+ strains. The addition of histidine in the medium allowed for better growth of the HDC+ strain but not HDC- strain. Addition of histidine also allowed for better acid resistance in all tested conditions (stationary phase and exponentially grown cells).
A HDC+ *Lactococcus lactis* strain containing a cloned HDC pathway of *S. thermophilus* CHCC1524 was compared with an HDC- strain carrying an empty vector in acid stress survival tests and growth experiments. Survival to acid stress was approximately 100 times higher for HDC+ cells than HDC- cells, but only in the presence of histidine. In growth experiments using rich medium +/-histidine, the HDC pathway had only minor effects on the growth rate of *L. lactis*. This suggests that the proton motive force generating effect of the pathway, although beneficial for the cells, has a smaller role in the selective pressure on maintaining the histidine decarboxylation pathway than the acid stress resistance mechanism.
HdcB of the HDC pathway. During characterization of the *S. thermophilus* HDC pathway it was found that maturation of HDC was strongly dependent on the presence of the *hdcB* gene that codes for a protein of unknown function. Deletion of *hdcB* resulted in reduction of histamine production. HdcB was shown to be involved in the maturation of HdcA. It catalyzes the cleavage of the π preprotein of HdcA into the α and β subunits that form the active complex.
Ornithine decarboxylation (ODC) and lysine (LDC) decarboxylation pathways. The physiological role of the ODC pathway was examined in *Lactobacillus* 30A from the gastrointestinal tract, *L. brevis* IOEB 9906 from molasses and *O. oeni* IOEB 89006 and S22 from wine. The LDC pathway of *L.* 30A was also investigated. Ornithine improved acid resistance and cytosolic pH homeostasis in *L. brevis* IOEB 9906, *L.* 30A and *O. oeni* S22. In

L. 30A the presence of lysine also improved acid resistance but to a much lower extent than ornithine and it did not contribute to alcalinisation of cytosolic pH contrary to ornithine. Remarkable differences were observed within the species *O. oeni.* In strain S22 acid shock resistance largely relied upon the action of the ODC pathway, whereas strain IOEB 89006 displayed a good "natural" acid resistance, which didn't appear to be influenced by the presence of ornithine. In conclusion the ODC pathway may improve acid resistance of LAB, but not in strains that already display a good intrinsic acid resistance. Moreover, the LDC pathway of *L.* 30A does not contribute significantly to acid resistance.

Tyrosine decarboxylation (TDC) pathway. The role of the TDC pathway was investigated in *L. brevis* strains 79B8c23 (TDC+) and NS25 (TDC-), in a *Sporolactobacillus* strain P3J (TDC+) and in *L. reuteri* strains NS98 (TDC+) and DSM20016 (TDC-). The presence of the TDC pathway in the different strains had no observable effect on growth. Only slight differences in tyramine production were observed at pH 3.5 and 4. However, the addition of tyrosine in the medium at pH 3.5 allowed for better growth of both the TDC+ (NS98) and TDC- (DSM20016) strains, suggesting that this behaviour is uncoupled with tyrosine decarboxylation. Moreover, the TDC+ NS98 strain also produced significant amounts of tyramine at pH 3.5 so the pathway was active and tyrosine was used for both growth and BA-production.

Agmatine deiminase (AGDI) pathway. The role of the AGDI pathway involved in putrescine-production was evaluated in *O. oeni* Be15 (AGDI+) and LJ0T1B (AGDI-). Screening for both growth evaluation and acid stress resistance did not reveal significant differences between the strains, indicating that the presence of the AGDI pathway had only a minor impact.

In addition the tyramine and putrescine producer *Enterococcus faecalis* V583 (TDC+, AGDI+) was selected for physiological characterization and knock-out derivatives unable to produce tyramine or putrescine or both BA were constructed. This work is still is progress.

Relation between BA-production and N-sources in wine

Peptides as substrate instead of free amino acids. To determine if LAB can produce BA from peptide precursors instead of amino acid precursors, the putrescine-producing strains *O. oeni* BR14/97 and IR BL0079 and the tyramine-producing strains *Lactobacillus plantarum* IR BL0011 and IR BL0076 were grown in a synthetic medium supplemented with peptides. Results showed that LAB can produce BA from peptides.

Impact of the nitrogen content of wines. The impact of nitrogen composition of wine on BA production was analyzed by using a synthetic must fermented by various yeasts that differ in terms of nitrogen requirements. The nitrogen composition of the resulting wine, particularly the amino acid composition, varies considerably with yeasts. Malolactic fermentation (MLF) was initiated by inoculating a putrescine-producing strain of *Oenococcus oeni* (ODC+). At the end of MLF, very low levels of putrescine were detected, indicating that the yeast strain used for alcoholic fermentation had no effect on putrescine production. The same experiment was repeated with *L. plantarum* IR BL0076 strain. Small differences in putrescine and tyramine production after MLF were observed depending on the yeast used. An increase in BA production was observed with the wild type yeast compared to the mutant yeast which avoids peptides accumulation in wine. BA production is higher with yeast consuming little nitrogen.

Factors inducing BA production in LAB

TDC/AGDI pathways. The effect of low pH and high ethanol concentrations was evaluated on TDC and AGDI gene expression in *L. brevis* IOEB 9809 under winemaking conditions.

Both TDC and AGDI genes were induced by low pH (pH 3.2) and high ethanol concentration (12% v/v). In contrast this expression is apparently unaffected by supplemented aminoacids.

In *E. faecalis* V583 (TDC+, AGDI+), the maximum growth was obtained in the presence of agmatine and tyrosine. Their presence in the media had also an effect on the final pH of the medium. Production of tyramine was induced by low pH; however, the production of putrescine was related with the growth phase, the highest expression being measured at the exponential phase. Growth experiments under controlled pH conditions suggest that putrescine production is not under pH control.

In *L. lactis* CHCC7244 (AGDI+) the highest pustrescine concentration was reached after 24 h growth in the presence of agmatine and correlated with a pH increase in the media. Growth on different carbon sources (glucose, lactose or galactose) at different concentrations showed that the agmatine effect on growth and pH is observed only when the carbon source is consumed.

In some *L. lactis* strains the AGDI cluster is interrupted by the presence of an insertion element (IS) and do not produce putrescine. The IS is located between *ptc* and *agd*I genes. It was checked by qPCR that after 200 generations in presence of agmatine more than 90% of the population had lost the IS and became putrescine producers. RT-PCR analyses showed that agmatine is required for the expression of *ptc* and *agd*I genes. Expression of the IS element is constitutive and abolishes expression of downstream genes. A correlation between a 5-nucleotides region located ustream aguR and the instability of the IS element was observed. Only in the strain carrying the 5 nt, *ptc* is expressed. The simultaneous expression, but in opposite direction, of *ptc* and IS could provoke the instability of the IS element.

Transcriptional expression of the AGDI pathway of *E. faecalis* was analyzed by cloning the *aguR* and *aguBDAC* promoters in fusion to the promoterless vector pTCV-*lac* (Figure 4). *E. faecalis* cells carrying P*aguR* or P*aguB* fusions were grown in LB medium in the presence/absence of agmatine, NaCl or high temperature. Agmatine induced expression of both promoters, whereas high salt and high temperature had no impact. These experiments suggest that agmatine is the specific signal for induction of the pathway in *E. faecalis*.

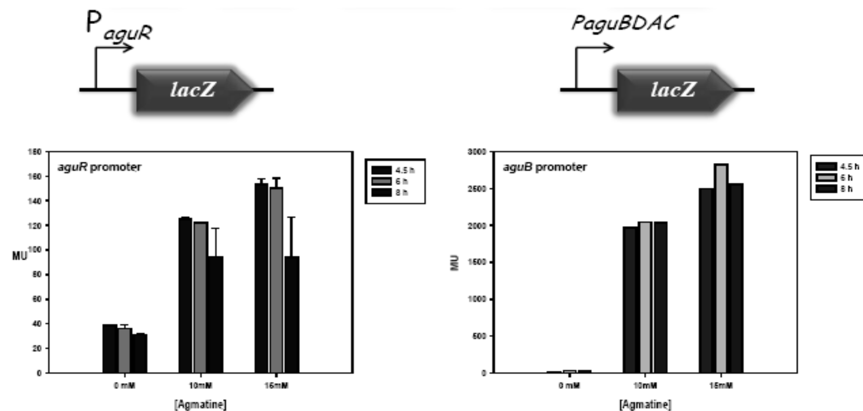


Figure 4. Transcriptional analysis of *agu* operons. On the top, we present the transcripcional fusion used in this work. On the botton the β-galactosidase activity (expressed in Miller Units) of the cells extract of *E. faecalis* carrying the P*aguR*-lacZ (left) or PaguB -lacZ (right) +/- agmatine.

ODC/LDC pathways. In *L.* sp. 30A two decarboxylases with alternate substrate specificity could be encountered (ODC and an LDC). Results showed cross induction phenomena of ODC and LDC by both ornithine and lysine. In *L. brevis* IOEB 9906 (ODC+), ornithine and lysine induced ODC activity, but they had no significant impact on LDC activity.

HDC pathway. The effect of histidine on *hdcA* expression was evaluated in *Streptococcus thermophilus* CHCC1524 by qRT-PCR. Results showed that histidine stimulates *hdcA* with a maximum induction reached at 6 mM. Northern blot analysis revealed the transcription of these genes as polycistronic mRNA including *hdcA*, *hdcP* and *hdcB.* There was no effect of pH and temperature on *hdcA* expression.

The influence of acidic stress on cell survival and histamine production was examined in *L. reuteri* DSM200016. No decrease of cell viability was detected after exposure to pH 6.5 to 4.1 and 50% survival was still observed at pH 1.8 (Figure 5A). *L. reuteri* produced histamine at all pHs tested. The optimal pH for histamine production ranged from 5 to 3 and efficient production was still detected when at pH 1.8. (Figure 5B), In summary, *L. reuteri* counteracts acidic stress very efficiently, and its HDC pathway is still active at very acidic pHs.

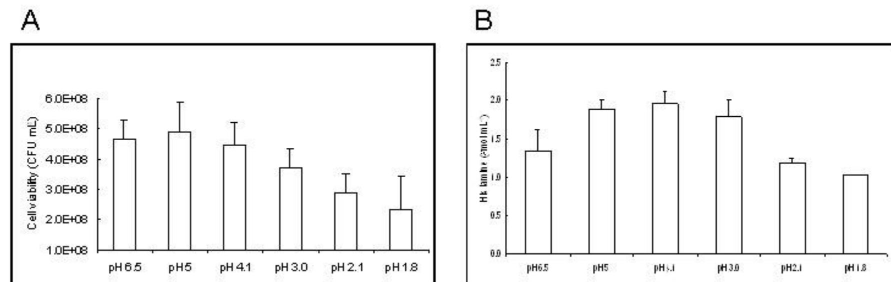


Figure 5. Analysis of influence of acidic stress on *L. reuteri* DSM200016. *Cell survival assessed by use of the LIVE/DEAD fluorescent kit (A). Histamine levels synthesized and secreted by 10^8 cells mL^{-1} are depicted.*

Work package 4. Physiology of BA-producing pathways: complementarity to citrate/malate degradation pathways

Physiological functions of BA-producing pathways and citrate and malate degrading pathways are believed to be metabolic energy generation and/or acid stress resistance. The two types of pathways may be exchanged to reduce the hazardous BA production in fermentation processes and replace it for the beneficial citrate/malate fermentations while still maintaining a healthy microbial flora. Successful experiments were performed to characterize the new OAD citrate degradation pathway in LAB and to determine the mechanism of induction of several pathways, and to unravel interaction between the expressions of the two types of pathways in the same organism.

The citrate and malate degradation pathways and putrescine producing pathway (from agmatine) were studied in detail in two microorganisms, *Lactococcus lactis* a starter LAB (SLAB) and *Enterococcus faecalis* a non-starter LAB (NSLAB). The results showed significant differences in the gene regulation depending on the microorganism studied. *L. lactis* showed transcriptional acidic activation for citrate metabolism and pyruvate metabolism, both involved in the production of aroma compounds. Whereas in *E. faecalis* decarboxylative pathways showed an exquisite regulation by the substrate and a delicate catabolic repression control mediated by PTS systems. For the BA producer (NSLAB, *E. faecalis*) none of the decarboxylative pathways were induced by acidification of the external growth medium.

The OAD system of LAB

Citrate metabolism in LAB is catalyzed by two distinct pathways. One pathways uses a soluble oxaloacetate decarboxylase, the other a membrane bound Na^+-pumping complex which is usually found in Gram-negative bacteria. The latter complex of *E. faecalis* was characterized. The studies indicated that the gene cluster involved in citrate degradation pathway in *E. faecalis* encodes a novel OAD subunit, called η subunit. Thus, the enterococcal OAD complex was proposed to be constituted by 4 subunits α, β, δ and η. In *E. faecalis*, the *Kpn*-α subunit is splited into two subunits, *Ef*-α and *Ef*-δ and we suggested the new subunit (*Ef*- η) has functional properties analogous to the *Kpn*-γ subunit a homologue of which is not encoded in the *E. faecalis* cluster. Specific interaction between α, δ and η were found by using

avidin resin (the δ subunit is biotinylated). The complex was identified not only in the soluble fractions of the *E. faecalis* but also in the membrane fraction suggesting it interacts with the membrane through the membrane bound β subunit (Figure 6).

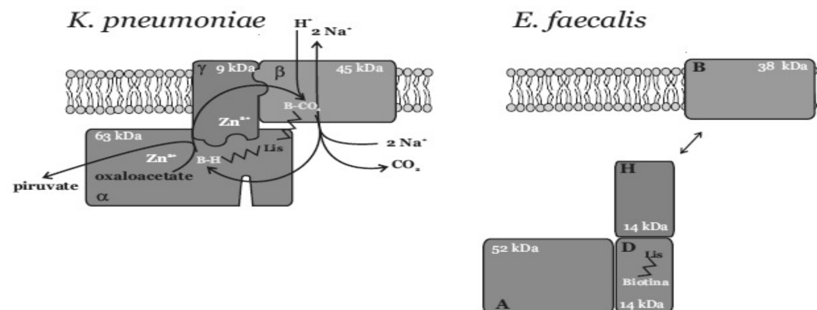


Figure 6. Membrane bound Oxaloacetate Decarboxylase Complexes found in *K. pneumonia* and *E. faecalis.*

Citrate and pyruvate metabolism in *L. lactis* CRL264. This strain is an aroma-producing organism used in starter cultures for the elaboration of dairy products. The species is essentially a fermentative microorganism, which cometabolizes glucose and citrate to yield aroma compounds through the diacetyl/acetoin biosynthetic pathway. The results showed that under acidic growth *L. lactis* CRL264 expresses coordinately the genes responsible for citrate transport and its conversion into pyruvate. The impact of acidic growth on glucose, citrate, and pyruvate metabolism of *L. lactis* CRL264 has been investigated by proteomic analysis. The results indicated that acid growth triggers the conversion of citrate, but not glucose, into acetolactate via pyruvate. Next, the influence of external pH on regulation of the diacetyl/acetoin biosynthetic pathway in *L. lactis* CRL264 was analyzed at the transcriptional level. Our results support that this biosynthetic pathway is induced at the transcriptional level by acidic growth conditions, presumably contributing to lactococcal pH homeostasis by synthesis of neutral compounds and by decreasing the levels of pyruvate.

Citrate metabolism in *Enterococcus faecalis.* *E. faecalis* plays a critical role in the manufacture of regional cheese and it is the main responsible of the presence of BA in this food. Bearing in mind that at the beginning of the present project there was little knowledge about the decarboxylative pathways in the non-starter bacteria; we started the characterization of the citrate metabolism in this microorganism. The first approach was a bioinformatic search of the genes that encode the citrate lyase complex. This complex splits citrate into oxaloacetate and acetate and is involved in all the known anaerobic bacterial citrate fermentation pathways. Although citrate fermentation in *E. faecalis* has been investigated before, the regulation and transcriptional pattern of the *cit* locus has still not been fully explored. To fill this gap, in this project we demonstrated that the GntR transcriptional regulator CitO is a novel positive regulator involved in the expression of the *cit* operons. Our analysis showed that two operons are specifically activated by the addition of citrate to the medium. A mutant strain with an interrupted *citO* gene was constructed, causing a total loss of the ability to degrade citrate. Furthermore, we present evidence that CitO binds to the *cis*-acting sequences O1 and O2, located in the *cit* intergenic region, increasing its affinity for these binding sites when citrate is present and allowing the induction of both *cit* promoters.

Acetoin biosynthesis pathway in *Enterococcus faecalis*. Enterococci can be used in the food industry as starter or probiotic cultures but also implicated in severe multi-resistant

nosocomial infections. Acetoin is an important physiological metabolite excreted by many microorganisms. The genes involved in acetoin biosynthesis in *Enterococcus faecalis* were identified and the transcription analysed. Two divergent transcriptional units were detected: *alsSD*, which codes for the α-acetolactate synthase (AlsS) and α-acetolactate decarboxylase (AlsD). Transcriptional analysis showed that *alsSD* operon levels are maximal during the exponential phase of growth and sharply decrease thereafter. Furthermore, it was found that *alsSD* operon is enhanced upon addition of pyruvate to the growth medium. An isogenic *als* mutant strain was constructed, this strain completely lost its capacity to use pyruvate as carbon and energy source in LB medium at initial pH 4.5. Our results suggest that these decarboxylation pathways give the enterococcal cells a competitive advantage in pyruvate rich medium during growth at low pH.

Malate metabolism in *Enterococcus faecalis*. The *E. faecalis* genome contains two paralogous genes, *maeE* and *citM*, coding for proteins that show high homology with members of the malic enzyme family. The putative proteins MaeE (41 kDa) and CitM (42 kDa) share a high degree of homology between them. However, the genetic context of each gene suggested that *maeE* is associated with malate utilization whereas *citM* is linked to the citrate fermentation pathway. The gene products were biochemically and physiologically characterized. Genetic studies in *E. faecalis* showed that the citrate fermentation phenotype is not affected by a deletion of *citM*. On the other hand, *maeE* gene disruption originated a malate fermentation deficient strain indicating that MaeE is responsible for malate metabolism in *E. faecalis.* Lastly, it was demonstrated that malate fermentation in *E. faecalis* is associated with cytoplasmic and extracellular alkalinization which clearly contributes to pH homeostasis in neutral or mild acidic conditions.

Transcriptional regulation by acid stress. Under acidic growth *L. lactis* CRL264 expresses simultaneously the genes responsible for citrate transport (plasmidic *citQRP* operon) and its conversion into pyruvate (chromosomal *citMCDEFXG* operon). The molecular mechanism of the activation was studied using as a model the acid inducible P*cit* promoter region isolated from *L. lactis*. This promoter region was selected to design an expression system for *L. lactis*, which is also functional in *E.faecalis*. Mutational analyses performed on the P*cit* region suggest that the region from -60 to -87 (related to transcriptional initial site) is involved in the activation of this promoter. Deletion of the proximal region to the – 35 primbow box (8 nucleotides), produced a loss of the activity of the promoter. Two direct repeats were identified in the P*cit* promoter region (DR1 and DR2), however, the mutations of these regions was not involved in the mechanism. Thus, the mechanism of the activation at low pH of the P*cit* promoter could involve the positioning of the upstream sequences in an optimal distance to the promoter by torsioning the DNA.

Hierachical regulation. The genes for catabolism of non preferential carbon sources were induced in *E. faecalis* by growth on the cognate substrate and their induction is controlled by transcriptional activation. In case of citrate CitO is required for the specific activation of the *cit* operon encoding the citrate transporter and the enzymes involved in the citrate metabolism. The activator *gntR* is a cytoplamatic factor that senses the presence of citrate and specifically bind to the intergenic region. For malate, a two component system (maeK/maeR) is required for the transcriptional activation of the malate transport (maeP) and malic enzyme (maeE) genes. MaeK is a kinase that senses malate outside the cells and phosphorylates maeR that binds to a cis-acting element localized in the intergenic region. The agmatine deiminase pathways is under the control of a membrane transcriptional activator AguR, which in presence of agmatine induce the *aguBDCA* operon.

Using genetic approaches the presence of multiple *cre* sites were identified in the proximal promoter region of the citrate fermentation *cit* cluster and in the intergenic region of the malate fermentation *maePE* region. A bioinformatic search showed that in the intergenic region of the promoters region of the putrescine producing *agu* cluster a highly homologous *cre* site was found. A complex catabolite repression system controls the expression of the activator CitO which reduces the uptake of citrate and also inhibits the expression of the catabolic genes required for citrate metabolism. Glucose repressed the expression of the malate degradation operon. The response to glucose on the putrescine operon suggested an independent CcpA mechanism involved in this regulation (Figure 7).

Figure 7. Transcriptional Regulation of the decarboxylative pathways characterized in *Enterococus faecalis*

Work package 5. Reduction of BA levels in foods by inhibiting BA-producing pathways and biodegradation of BA

In many fermentation processes, the composition of the microbiota cannot be controlled at the beginning of the food chain, due to the fact that production practices are non-sterile. In fact, a specific, non-starter flora coming with the substrate (milk, grapes, etc) may be essential for the particular fermentation product. Then, BA-producers are likely to enter the food chain as non-starter LAB that are indigenous to the natural starting material. Work package focused on damage control in those cases where the presence of BA producers in the fermentation process is a fact. Two basically different strategies are followed both aiming at reduced accumulation of BA in the products. One strategy aims at preventing the formation of BA by searching for (food-grade) compounds that inhibit the BA producing pathways. The other strategy allows the formation of the BA by the organisms and searches for LAB that, subsequently, are capable of degrading the BA again. The former approach requires the availability of inhibitors, that are acceptable from a consumer point of view and do not change the properties of the products significantly. Inhibitors of the histamine producing pathway were identified and modeling studies for the rational design of inhibiters were initiated. The recent description of LAB strains with a GRAS status that are capable of catabolizing BA allows for the degrader approach. The identification of a number of strains capable of degrading different BA and their ability to degrade BA during fermentations is one of the most promising results of the BIAMFOOD project.

Inhibitors of BA producing LAB

Inhibition of the histamine pathway. *Lactococcus lactis* strain expressing the histidine/histamine exchnager *hdcP* of *Lactobacillus hilgardii* 0006 was used for studies on

the inhibition of histidine transport via HdcP by histidine analogues. Histidine transport by the endogenous proton symporter LysQ of *L. lactis* was used as a control. A number of analogues with different substitutions/additions at the histidine side chain as well as at the α-amino group and α-carboxyl group were tested, resulting in histidinol as an inhibitor of HdcP, which does not interfere with histidine uptake via LysQ. Histidinol also inhibited significantly the histamine production by a recombinant strain of *L. lactis* expressing the complete histidine decarboxylation pathway from a histamine producing *Streptococcus thermophilus* strain. In conclusion, histidinol is a potential inhibitor of histamine formation in fermentations, but also can serve as a model for the design of new inhibitors that may be applied in industry.

Rational design of inhibitors of BA producing pathways. The histidine/histamine exchanger HdcP in the histamine producing pathway found in LAB is a member of the APC superfamily of secondary transporters. Averaged hydropathy profiles of APC families are very similar to the profiles of a number of unrelated transporter families for which a 3D crystal structure is available suggesting a similar fold for the former. Alignment of the family hydropathy profiles allowed for the construction of a homology structural model for the HdcP family of transporters. Meanwhile, the similar fold has been confirmed by a report on the structure of the arginine/agmatine transporter AdiC of *E. coli*, a homologue of HdcP. This allows for the construction of a much more detailed 3D model by homology modelling through threading. Using the model, a range of residues possibly involved in substrate binding in HdcP have been assigned. In order to confirm the role of residues in histidine or histamine binding, functional analysis of single mutants was performed. To assess which functional groups of the histidine and histamine molecules are important in the recognition by HdcP, a number of histidine analogues were tested for ability to compete with histidine for transport. Although the results inform about the substrate recognition, the 3-D model needs to be improved for a better interpretation and subsequently a rational strategy for site directed mutagenesis.

BA-degrading LAB strains

Screening of strains. Different types of cheeses that use to have high amounts of BA were analyzed for the presence of BA degrading bacteria. For this purpose, screening protocols to detect tyramine and histamine degrading LAB in cheese were developed. Isolated colonies from the screening were grown in liquid medium with the corresponding BA and its degradation was checked by HPLC. Different percentages of degradation were obtained (from 30% to 85%). Based on their capability to degrade histamine or tyramine, as well as the absence of *tdc* and *hdc* genes and their ability to grow on milk, 17 strains were selected and identified by sequence of the 16S rRNA as *Lactobacillus casei.* All the selected strains were sensitive to the tested antibiotics (tetraciclyne, erythromicyne and chloramphenicol) and were grouped in eight different plasmidic profiles. Since all the selected strains, independently of the cheese sample or the BA used for the enrichment rounds, were identified as *L. casei,* the capacity of all of them to degrade both, tyramine and histamine was evaluated. The strains 4a, 5b, 16b, 18b and 39b, which showed better ability to degrade tyramine and/or histamine, were selected for further experiments.
A selection of organisms from the Chr. Hansen Culture Collection was also analyzed for their ability to degrade histamine. The organisms screening consisted of 15 *Micrococcus* sp., 12 *Corynebacterium* sp. and 4 *Brevibacterium linens* strains. The isolates were grown overnight at 30°C with shaking in 50 ml Brain Heart Infusion (BHI) broth. The cells were then harvested and washed and resuspend in 5 ml 0.05 M phosphate buffer, pH 7 supplemented

with 0.54 mM histamine. The cell suspension-histamine mixture was then incubated aerobically for 2 days at 30ºC. After incubation the mixture was centrifuged, and the histamine content measured in the supernatant using a simple ELISA screening kit and GC-MS. One isolate, *Brevibacterium linens* CHCC 3811 was found to be able to degrade histamine.
In addition to the originally planned search for degraders isolated from cheeses, a collection of *L. plantarum* strains isolated from red wines was also screened as an extra task. The strains were grown in liquid medium with the corresponding BA and the degradation rate checked either by TLC (Thin Layer Chromatography) or HPLC analysis. Two strains (named Lp1 and Lp2) able to degrade putrescine and tyramine were identified and analysed in combination (coinoculation) with *Enterococcus faecium* (tyramine producer) and *Lactobacillus brevis* (putrescine producer). A 30 % reduction in tyramine concentration was obtained in the presence of *L. plantarum* Lp1 and a 40% reduction in putrescine concentration in the presence of *L. plantarum* Lp2 . The latter *Lactobacillus plantarum* strains able to degrade tyramine and putrescine were used in microvinification trials.

BA-degradation in a cheese model. A Cabrales-type mini cheese model was optimized to check the degrading strains before to include them in a real fabrication. The selected strains were used as adjunct starters at two different concentrations (10^3 and 10^6 cfu ml^{-1}) in the presence of tyramine (*E. faecalis* or *E. durans*) or histamine (*Lb. buchneri* or *Lb. parabuchneri*) producers. BA producer strains evolution was followed by qPCR and BA concentration was determined by HPLC analysis. The results with *E. faecalis* BA62 as tyramine producer in the presence and absence of *Lb. casei* 5b is shown in figure 8. A 100% of reduction on tyramine accumulation in the presence of BA degrader strains, either inoculated at 10^3 or 10^6 was observed.
The results with *Lb. buchneri* as hystamine producer in the presence and absence of *Lb. casei* 5b are shown in figure 9. Histamine accumulation was around three times reduced. The best results were obtained with *Lb. casei* 4a and 5b, so these strains were selected for the cheese trials of WP8.

Figure 8
(TMA=tyramine)

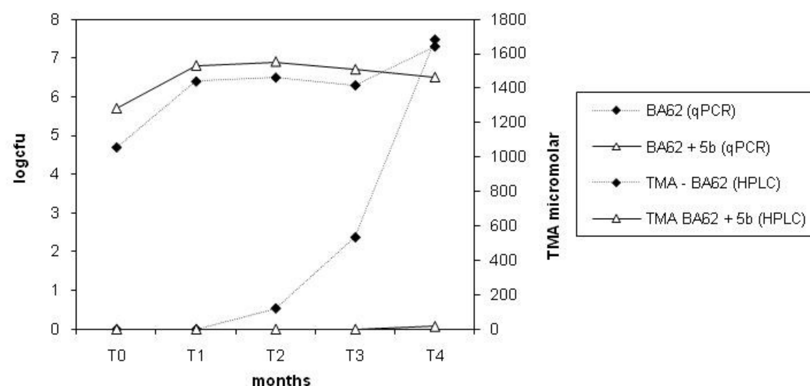
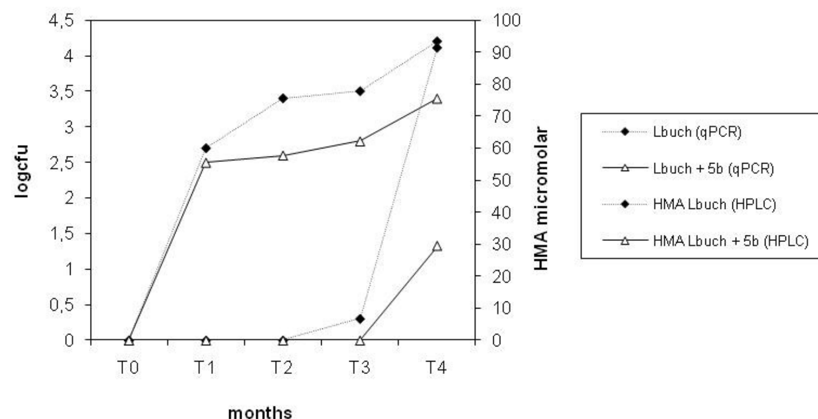


Figure 9
(HMA=histamine)



Histamine degradation pathway

The exact pathway in which histamine is degraded is unknown, and three possible pathways have been proposed; (i) conversion to imidazole acetaldehyde via a histamine oxidase, (ii) conversion to glutamylhistamine via a glutamylhistamine synthase and (iii) conversion to methylhistamine via a histamine methyltransferase. Samples were analysed using a GC-MS triple quadropole in order to elucidate the pathway. Silylation dervivatization was used to react with hydroxyl, carboxylic acid, amine, thiol and phosphate functional groups in the samples. The degradation of histamine by *Brevibacterium linens* CHCC3811 was confirmed by this method, using the non-histamine degrading strain *Brevibacterium linens* CHCC3812 as negative control. However, despite using this powerful analytical method, it was not possible to identify the histamine degradation products. The same sample set was also run on a MS-QTOF, which also failed to identify the degradation products. Other analytical techniques such as NMR or HPLC with radiolabelled substrates may be required in order to study this pathway in greater detail.

Work package 6. BA-producers- gastrointestinal tract interactions

A possible health threat of foods could be the presence of BA-producing organisms, if they survive the passage through the digestive tract. The objective of this work package was to investigate in model systems the performance of BA-producing LAB in the gastrointestinal environment as well as the influence of the BA and their producers on human cell lines. Selected BA-producing and non-producing isogenic LAB isolated from fermented foods were analyzed for viability under conditions mimicking the conditions in the human GI track in a model that simulates the *in vivo* digestive tract conditions.

Gastrointestinal stress

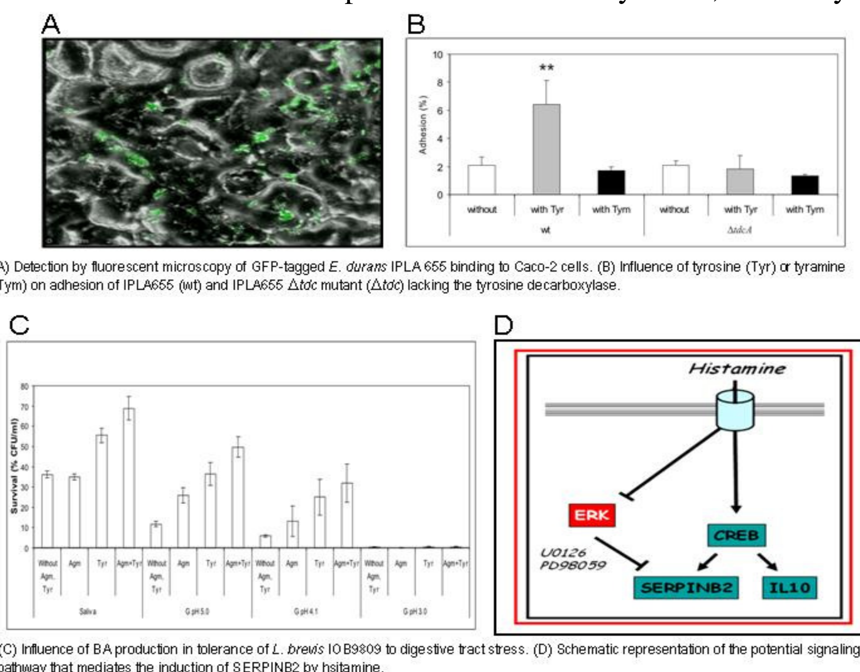
GI model system. An *in vitro* model system was developed to monitor the passage and survival of BA producing LAB in the GIT, and validated with lactic acid bacteria (LAB) and *Bifidobacteria* strains. This model simulates the normal physiological conditions of the GIT, including the presence of lysozyme and pepsin, sequential gastric emptying at increasingly-lower pH values, and the presence of bile salts and pancreatin. Moreover, during the development of the project the model has been modified to adapt to the various levels of resistance of the LAB analyzed.

Survival under GIT stress. LAB isolated from cider, cheese, meat, fish and human gut were tested for survival under low pH and anoxic conditions using synthetic media. Of these, three BA- producer strains were selected for testing in the *in vitro* model: the dairy tyramine-producer *Enterococcus durans* IPLA655, the wine tyramine- and putrescine-producer *Lactobacillus brevis* IOEB9809, and the histamine-producer *Lactobacillus reuteri* DSM20016 isolated from human intestine. The three bacteria were able to produce BA under GIT stress conditions, when their respective precursors were present. However, a beneficial effect of BA precursors on cell survival to gastric stress was detected only for *Lactobacillus brevis* IOEB9809 (Figure 10C). Moreover, a transcriptional co-activation of the two BA biosynthetic pathways, accompanied by an increase of tyramine production, was observed. The overall results show that the resistance to GIT challenge of LAB is species specific, that BA production contributes to GIT tolerance of LAB sensitive to these stresses and that histamine,

tyramine and putrescine could be produced in the GIT by LAB. Therefore, these bacteria could contribute to BA content in the host. This contribution would be greater if such strains were able to colonize the gut and continue to synthesize BA.

BA-producers – human cell line interactions

Adhesion of BA-producers. Adhesion of bacteria to intestinal cells can be correlated with transient colonization of intestinal cells, which could be a factor for their immunomodulation. The secretion of the pro-inflammatory cytokines such as IL-6, IL-8 and TNF-α is a hallmark of the inflammatory response to pathogens in the intestine and one of the potential benefits of probiotic bacteria is the suppression of this inflammatory process by inducing the anti-inflammatory cytokines such as IL-10. ELISA assays to test levels of cytokines produced by Caco-2 epithelial cells and macrophages in the presence of LAB and Bifidobacteria were standardized. Moreover, the technique was applied to determine levels of cytokines in supernatants of adhesion assays performed with Caco-2 cell lines and LAB. Although these bacteria showed a substantial adhesion, no significant increase in the production of IL-6 and IL-8 cytokines was observed, suggesting that they do not trigger an overt inflammatory response in human intestine epithelial cells. In addition, the ability of wine LAB to immunomodulate macrophages has been investigated (Fernandez de Palencia et al. 2009). The results showed that production of IL-6 and IL-8 is affected by the presence of the bacteria. The three BA-producers mentioned above were analyzed for binding ability to Caco-2 cells. All BA-producers were able to bind efficiently to the epithelial cells (3-40%), with *L. reuteri* DSM20016 binding most. All were also able to produce BA in these conditions. Moreover, an influence of the tyramine biosynthetic pathway on binding ability was only observed for the tyramine producer *E. durans* IPLA655. When tyrosine was present in the adhesion assay, a significant increase (approximately 3-fold) in its adherence to Caco-2 cells was observed (Fig. 10A,B). In contrast, the presence of tyrosine did not affect the binding of the isogenic *E. durans* IPLA655 Δ*tdcA* mutant strain, which is unable to produce tyramine. HPLC revealed that in the presence of 10 mM tyrosine, the dairy



(A) Detection by fluorescent microscopy of GFP-tagged *E. durans* IPLA 655 binding to Caco-2 cells. (B) Influence of tyrosine (Tyr) or tyramine (Tym) on adhesion of IPLA655 (wt) and IPLA655 Δ*tdc* mutant (Δ*tdc*) lacking the tyrosine decarboxylase.

(C) Influence of BA production in tolerance of *L. brevis* IOB9809 to digestive tract stress. (D) Schematic representation of the potential signaling pathway that mediates the induction of SERPINB2 by hsitamine.

Figure 10.

(cheese) strain was able to synthesize tyramine (1.4 x 10^7 bacteria produced 141 nmol of tyramine per ml in 1 h). Supplementing the assay with 140 μM tyramine did not affect the binding of either strain to Caco-2 cells (Fig. 10B). These results suggest that activation of the tyramine biosynthetic pathway, rather than the actual production of tyramine, could be involved in this enhancement of adhesion. Production of the pro-inflammatory cytokine TNF-α by Caco-2 cells after exposure to the *E. durans* wild-type and mutant strains in the presence or absence of 10 mM tyrosine was determined. In the absence of tyrosine, the presence of either strain did not significantly affect the levels of the cytokine secreted by the Caco-2 cells. In the control samples lacking bacteria, the presence of tyrosine resulted in a 2-fold decrease in TNF-α levels, which was accompanied by consumption of 83.5% of the tyrosine (Fernández de Palencia et al., 2011). Significantly lower levels of this cytokine (8% and 3.8% of the levels with the mutant strain and the control, respectively) were detected in the presence of the wild-type strain than in the presence of the mutant or the control when tyrosine was included in the assay. The production of tyramine was confirmed in the wild-type strain samples, where it reached a concentration of 3 mM in the presence of 10 mM tyrosine. Moreover, a similar level of tyrosine (approximately 4.3 mM) was detected in samples exposed to both strains, indicating that the differences in cytokine levels provoked by the bacteria were not due to differences in the availability of tyrosine to the Caco-2 cells. Therefore, the reduction in the synthesis of TNF-α by the wild-type strain could be associated with the tyramine biosynthetic pathway. The overall results indicate that *E. durans* IPLA655, present in cheese, could survive in the intestinal environment and synthesize tyramine in the colon, using this ability as a survival and colonization mechanism that enhances adhesion to the intestinal epithelium and reduces Th1 activation of the immune system (Fernandez de Palencia et al., 2011).

Immune response. Gut epithelial cells are a component of the innate branch of the immune response, and these results therefore suggest an effect of BA-producers on innate immunity. However, their influence on the adaptive side of the immune system still remained to be evaluated. Therefore, the effects of biogenic amines (BA) on human monocyte-derived dendritic cells and macrophages were determined using the following parameters: expression of cell surface maturation markers, release of pro-inflammatory and anti-inflammatory cytokines, and expression of inflammation-associated gene markers. Histamine, putrescine and tyramine were found to increase the expression of maturation markers on human dendritic cells, with histamine exhibiting the highest maturation-inducing ability. This result was confirmed upon analysis of the cytokines released by dendritic cells, when histamine triggered the secretion of significant levels of IL-12p40 in a dose-dependent manner. Moreover, BA increased the IL-10/IL-12p40 cytokine ratio in response to LPS, implying that they down-modulate the pro-inflammatory action of LPS, and suggesting that exposure to BA might lead to diminished immunogenic or inflammatory responses to the pathogen and pathogen-derived products contained in traditional food fermentations.

Based on these results, the influence of BA-producers on the gene expression profile of human macrophages was approached by determining the effect of histamine and tyramine on the expression levels of macrophage polarization markers by the use of custom-made microfluidic gene cards. Analysis of 54 macrophage polarization-related genes (including 21 specific for anti-inflammatory macrophages and 12 for pro-inflammatory macrophages) revealed that histamine enhanced the expression of the *SERPINB2* anti-inflammatory gene in human macrophages. In line with this result, histamine was found to collaborate with ERK inhibition in promoting higher *SERPINB2* expression levels, suggesting that its effects could be mediated through inhibition of the ERK signalling pathway. In terms of signalling, histamine was found to cause a transient reduction in the level of activation (phosphorylation)

of ERK, as well as a transient increase in the phosphorylation (activation) of CREB., in agreement with the positive action of CREB on *SERPINB2* expression. Therefore, histamine, and possibly tyramine, modifies the gene expression profile of human macrophages, and the modification by histamine correlates with a decrease in ERK, and an increase in CREB, activation (Figure 10D).
The analysis of the effect of histamine was finally analyzed in the context of medium conditioned by *L. reuteri* DSM200016, whose growth in the presence of histidine results in the generation of large levels of histamine. Exposure of macrophages to bacteria-conditioned medium revealed that MRS medium, by itself and in the absence of bacterial growth, dramatically up-regulates *SERPINB2* expression, thus preventing this type of assay from providing significant results. Therefore, in future, this type of assays must be carried out after bacterial culture in a medium that does not modify the macrophage gene expression profile (e.g., RPMI 1640).

Work package 7. The '*Oenococcus oeni* project'

The genome sequence of the wine bacterium *Oenococcus oeni* is available in the public domain. Previous attempts have shown that the membrane proteins of *O. oeni* are very recalcitrant to heterologous expression which is a bottleneck in the study of the BA-producing and citrate/malate degrading pathways in the organism. In view of the importance of *O. oeni* in wine making, different approaches were taken to overcome this technological hurdle. The genes encoding the putative transporters for citrate and malate were cloned in *Lactobacillus plantarum,* a closely related organism, however, without success. Several approaches were taken to make *O. oeni* accessible for genetic manipulation. In a more brut force approach homologues the citrate and malate transporter family from different organisms were heterologously expressed resulting in a number of functionally expressed transporters. Finally, the membrane proteome of *O. oeni* was studied by mass spectrometry.

Transformation of **Oenococcus oeni**

Molecular studies in *O. oeni* have been obstructed owing to the lack of appropriate techniques for DNA manipulation in ths species. The combination of several electroporation parameters with the usual wall-weakening agents, such as glycine, lysozyme or penicillin, used for the electroporation of Gram-positive bacteria was unable to make the cells permeable without compromising their survival.
We succeeded when we used ethanol which is known to increase membrane fluidity in many bacteria. Hence, a transformation system was developed successfully for *O. oeni*. The next step was the design of a suicide vector to delete genes in *O. oeni*. However, this suicide vector was used to obtain malate and citrate mutant transporter by single homologous recombination without success. Thus another strategy was designed. A suicide vector, derivated from pGIDO52, was constructed by deletion of the entire region implied in the Gram positive replication mechanism. The 1.9 kbp vector obtained was named pERY and would be unstable in *Oenococcus oeni* strains. A multiple cloning site was added into the pERY vector to allow cloning of an internal region of target genes for homologous recombination. However, no mutants were obtained by using pERY. In a last attempt, it was decided to delete genes by double homologous recombination: In fact the nonoccurrence of single cross over recombination could be explained by the cleavage of the circular DNA by a host-controlled restriction system. So linearization prevents integration of the circular DNA into the

chromosome. An alternative strategy was to disrupt genes by double homologous recombination. *MaeP* and *mleP* (malate/citrate permease) were cloned into this suicide vector, but no positive clone was obtained.

Heterologous production of **O. oeni** *membrane proteins*

The transporters believed to be involved in the citrate and malate degrading pathways in *O. oeni* - and therefore of interest for wine making - are members of the Auxin Efflux Carrier family. To further investigate the AEC family of transporters in relation to the BIAMFood project, a strategy was followed involving the cloning of multiple AEC family members of *O. oeni* and other Gram-positive bacteria, identified on basis of BLAST search results. From *O. oeni*, 5 genes, including *mleP* and a gene located in a putative citrate metabolism cluster, were selected. From other Gram-positives, two genes were selected from *Lactobacillus brevis*, two

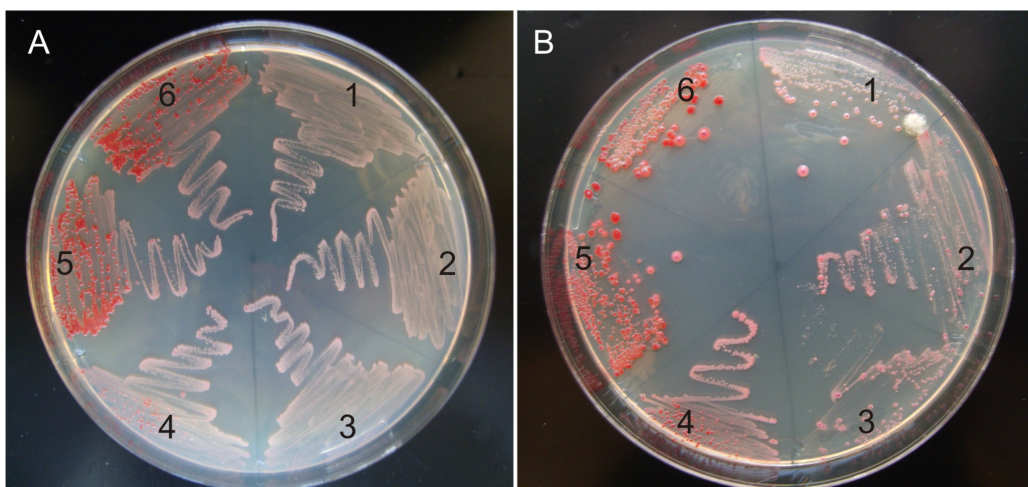


Figure 11. Malate fermentation plate assay. *L. lactis* NZ9000 (lacking a malate transporter) harboring plasmids for nisin inducible expression of (1) SACOL_2248, (2) OEOE_1197, (3) OEOE_0419, (4) LVIS_0568, (5) CPE0529 and (6) SMU.138 was streaked on plates containing 2,3,5-triphenyl tetrazolium choride. Red coloring indicates alkalinization due to malolactic fermentation. (A) Plate without nisin. (B) Plate with 5 ng/ml nisin. The red color in strain 5 and 6 in panel A shows that the nisin inducible promoter P_{nisA} in pNZ8048 allows for low level expression in absence of nisin.

from *Staphylococcus aureus*, one from *Streptococcus mutants* and one from *Clostridium perfringens*. Cloning was performed in two ways. For the purpose of functional expression and characterization, the genes were cloned in the NICE system expression vector pNZ8048 for inducible expression in *Lactococcus lactis*. For purification and/or detection of the proteins, histidine tag encoding sequences were included in the PCR amplified genes. Secondly, genes were cloned into *Escherichia coli* expression vectors derived from pBAD24 using a ligation independent strategy allowing for high throughput cloning. The vectors contained either the *gfp* or *phoA* gene, encoding green fluorescent protein (GFP) and alkaline phosphatase (AP), respectively, resulting in C-terminal GFP or PhoA translational fusions of the proteins. The reporter genes were used to estimate the level of expression and the localization of the C-termini of the cloned genes in the cytoplasm or periplasm.

Heterologous expression of SMU 138 of *S. mutans,* a close homologue of the malate transporter MleP of *O. oeni* in *L. lactis* NZ9000 that lacks a functional malate transporter allows for the conversion of malate into lactate. Such conversion resulted in alkalinization of the medium which could be visualized by a plate assay (Figure 11). Similar results were obtained with CPE0529 of *Clostridium perfringens* while the other putative genes encoding AEC family members failed to convert malate into lactate in *L. lactis* NZ9000. The SMU138 and CPE0529 proteins could be purified using the His-tag strategy. The failure to obtain functional expression of *O. oeni* transporters is not pertinent to the AUXIN family proteins.

The next step was to look at the expression and membrane topology of the AEC family transporters in the distant Gram-negative host *E. coli.* Alkaline phosphatase is a reporter of a periplasmic localization in this organism, while GFP reports on a cytoplasmic localization. Therefor, the two reporters are complementary. A high alkaline phosphatase activity was observed for cells expressing OEOE_1563 and OEOE_1466 of *O. oeni* and SMU.138 of *S. mutans* indicating expression of the proteins in the membrane and a periplasmic localization of the C-terminus. In agreement, GFP fluorescence of the corresponding GFP fusions was low. The periplasmic localization of the C-terminus is in agreement with membrane topology predictors. All other cloned transporters resulted in both a low alkaline phosphatase activity and GFP fluorescence, indicative of lack of expression. It is concluded that

Oenococcus oeni ***membrane proteomics***

The aim of the proteomic approach was the analysis of the membrane proteome and identification of the proteins. It was important to optimize the preparation of membrane vesicles from *O. oeni* ATCC-BAA1163 prior to 2D analysis. The first analysis has shown that metabolic proteins were observed. For this reason, a comparative analysis of the membrane and cytosolic proteomes was performed. This strategy allowed to identify a total of 92 spots belonging only to the membrane proteome of *O. oeni*. The protocol has been further improve by including several steps: treatments for de-lipidation with TFE/CHLF (2/1) and protein solubilisation with ASB-14 generating Type II membranes. These membranes were subjected to 2D-gel fractionation. This analysis revealed 21 spots that were identified. Among them, the existence of three new hypothetical proteins and their isoforms was validated. In addition, subunits of two ABC transporters involved in oligopetides and phosphate transport were identified. In summary, 225 peptides of the *O. oeni* ATCC-BAA1163 proteome has been identified and classified in the following functional classes: metabolism and energy production and conversion (40.2%); post-translational modification, protein and peptide turnover and chaperons (11%), translation, ribosomal structure and biogenesis (8.5%); membrane transporters and synthesis (7.3%); cell wall, membrane and envelop biogenesis (6.1%); cell division and chromosome partitioning (4.9%) and other functions including hypothetical proteins (17.1%).

Work package 8. Technological implementation and demonstration activities

Fermentation processes for different products like cheeses, wine and cider are incomparable. The presence in fermented products of LAB carrying BA-producing pathways not neccessarily imply the presence of BA in the final product. The objective of this work package was to carry the laboratory results to the industrial fermentation processes for validation and effectiveness. Small scale fermentations were performed making cheese, wine and cider in close collaboration with the producing beneficiaries. Several issues from the other work packages were addressed, like the presence of pathway inhibitors, novel BA degrading adjunct cultures, impact of salt on BA production, different oenological practices, the presence or absence of starters. Most promising finding: BA degrading adjunct cultures almost completely eliminate the presence of BA in Cabrales cheese.

Cheese trials

Impact of tyrosine transporter inhibitors on the formation of tyramine. Cheddar cheese trials were conducted in pilot scale in order to determine whether it was possible to inhibit the formation of tyramine through the use of specific tyrosine transporter inhibitors. A wide range of different inhibitors were identified in WP5, but for cheese application studies, only the food grade inhibitors, phenylalanine and hydroxyphenyl propionic acid were used. These compounds were added to the cheese milk at a concentration of 1 mM, and Cheddar cheese manufactured in the normal fashion. In order to provoke the production of tyramine in the cheese, tyramine producing organisms *Lb. curvatus* and *Lb. brevis* were added to the cheese milk. The cheeses were subsequently ripened for 6 months, and the level of tyramine measured periodically over the period. None of the inhibitors was shown to be effective in reducing or inhibiting the formation of tyramine in the cheese. Despite the fact that the inhibitors were added at the highest addition rate possible (due to solubility issues), they were probably present at too low a concentration to be effective in the cheese matrix. The identification of more potent food grade inhibitors would be of interest in order to further investigate this route of inhibiting BA formation in cheese.

Novel BA degrading adjunct culture for Cheddar cheese and/or Cabrales cheese. Cabrales cheese produced in the Asturias region of Spain is frequently found to have high levels of BAs present, and therefore it was used as a screening source for the isolation of BA degrading organisms. A number of BA degraders were isolated, and the most effective ones (*Lb. casei* isolate 4a and *Lb. casei* isolate 5b) were produced as freeze-dried cultures for cheese trial experiments. The strains were used to inoculate 350 liters of milk, and Cabrales cheese was manufactured in the normal way. After 1 month of ripening, the BA content of the cheeses was analyzed. There was a 100 % reduction of tyramine and about a 90 % reduction of histamine in the cheeses which clearly demonstrates that these BA degrading strains can be used to control the BA production in Cabrales cheese. However, it is believed that oxygen is necessary for the degradation to occur, and consequently such an approach may not be functional in anaerobic cheeses such as Cheddar and Gouda. Identification of the degradation products is an important element that needs to further investigation. In addition, the affect that reduction of BAs on the organoleptic properties of the Cabrales cheese needs to be ascertained.

Impact of salt reduction on the formation of histamine. During the last number of years it has become evident that *"low or reduced salt"* is another new major heath trend within the cheese market. High dietary intake of salt (NaCl) has been associated with an increased risk of hypertension. In order to reach this target, food producers are being encouraged by the regulatory authorities to formulated foods with reduced levels of salt. However, the affect of reducing salt in cheese may be critical regarding a possible increased risk of biogenic amine formation. Cheddar cheeses were produced in pilot scale in which the salt was adjusted to four levels (very low, low, medium and high). In order to provoke the production of histamine in the cheese, the histamine producing organism *Lb. parabuchneri* was added to the cheese milk. The cheeses were ripened for 9 months and the histamine levels were measured throughout the course of the period. Histamine was detected in all the cheeses at similar levels regardless of the salt concentration. This was a surprising result, as the expectation was that cheeses with very low and low salt levels would have significantly higher levels of histamine present. However, the level of histamine in all cheeses were extremely low (20-25 ppm) compared to what has been found in some commercial cheeses samples (1000-1500 ppm). Too low a concentration of free amino acids in the trial cheeses probably explains why no significant effect of salt on histamine production was evident. Additional trials in which a *Lb. helveticus* is used in order to boost the free amino acid level and improve the dynamic level of the experiment would be required in order investigate this aspect further.

Wine trials

Wines produced in the presence and absence of BA degrading LAB. Wines were produced in the Apulia region of Italy to which a tyramine degrading strain, *Lb. plantarum* Lp1 was added to the wine must. In order to secure the production of tyramine, a tyramine producing *Entercoccus faecium* was also added. The level of tyramine in the wine was subsequently measured. Indeed, the level of tyramine was reduced in the wine to which the degrading strain was added, but the reduction was by only about 15 %. A similar trial was conducted in French wines, in which BA degraders were also added to the wine must. In this instance, no reduction in the BA level was observed, probably due to the fact that the BA degrading strains were not pre-conditioned for wine conditions (*i.e.* low pH and high ethanol). A further factor in the degradation of BAs is that oxygen appears to be necessary. Also, the compatibility of the BA degrader with the malolactic fermentation culture need to be carefully considered with this approach.

Correlation between oenological practices and BA levels in wine. There is a range of oenological practices that may influence the production of BAs in wine. For example the addition of nitrogen to the wine must was investigated. Such addition of nitrogen is a common oenological practice in order to stimulate the growth of *Saccharomyces cerevisiae* which is essential for the alcoholic fermentation. However, it was shown that in French wines of the Rhone area that such a practice is undesirable as it results in higher histamine levels in wine produced with Syrah or Grenache grapes. Aging on lees is sometimes used in the wine industry in order to increase wine complexity and it is known that this practice results in an increase in the free amino acid level in the wine. The effect of this practice was investigated in wines produced in the Burgundy region of France. After the malolactic fermentation the levels of tyramine and putrescine were measured (Figure 12)

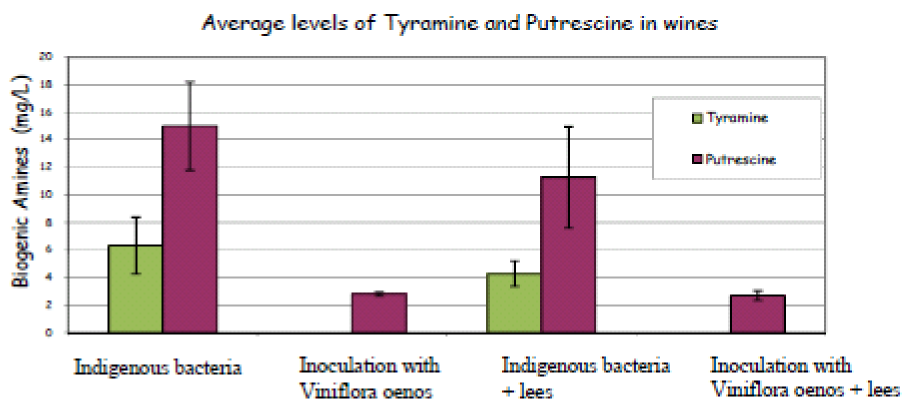


Figure 12.

Contrary to what has been described in the literature the level of BA in wine aged on lees was not higher than the control. When a commercial malolactic fermenting starter was added, the level of BAs was significantly reduced for putrescine, and in the case of tyramine, it was completely absent regardless of whether the wine was aged on lees or not. Sulphite addition is routinely used in the wine industry in order to stabilise the wine after the malolactic fermentation is complete. Interestingly, it was shown that it did not give protection against the formation of histamine or tyramine in wines aged over a period of 6 months.

Development BA-producers in wine inoculated with/without malolactic starters. Malolactic cultures are used in the wine industry in order to ensure a rapid conversion of malic acid into lactic acid. This conversion results in a dramatic improvement in the organoleptic qualities and stability of the wine. Another effect of using malolactic cultures is their role in controlling BA formation. In wines produced in the Bordeaux region of France, the malolactic starters S14, S28 and S161 significantly lowered the level of putrescine present in the wine. However, there was a clear correlation between the duration of the malolactic fermentation and the amount of putrescine accumulated. Therefore, fast malolactic fermentation is essential for BA control in wine. In wines produced in Italy, these results were confirmed for putrescine reduction when a malolactic starter was used, however, no reduction in histamine and cadaverine was observed. It appears that when BA producing organisms are present in the wine must that the only currently available solution for limiting the level of BAs in wine is to use malolactic starters.

Cider trials

Development of BA-producers in cider inoculated with/without starters. Cider is another fermented product in which BAs can be produced. In order to investigate this further a number of BA producers (2 putrescine producers, 1 tyramine producer and 1 histamine producer) were added to apple must from the Normandy region of France. Analysis of the cider showed that those ciders to which the BA producers had been added had approximately twice the level of BAs present compared to the control. Putrescine was the main BA present in cider, and by using a specific qPCR assay developed in WP2 it was possible to show that its production was mainly due to presence of ornithine decarboxylase positive populations.

Impact

The key concept underlining the social relevance of the BIAMFOOD project is 'traditional'. Traditions are 'repeated pattern of behaviors, beliefs, or enactment passed down from one generation to the next. Traditions are culturally recognized and sustained' (Folklore: An Encyclopedia of Beliefs, Customs, Tales. 1997). Hobsbawm (1983), distinguishes three well defined distinctive functions of traditions: a) establishing or symbolizing social cohesion and collective identities, b) establishing or legitimatizing institutions and social hierarchies, and c) socializing people into particular social contexts. Or, there is an understanding that a tradition is important with linking the past to the present as a form of identity making, with a particular importance for the social importance of the tradition-bearers. Tradition entails a complex set of relationships between the past and the present, in which traditional food productions and consumptions have a noticeable role (Holtzman, 2006). 'Fermentation is one of the oldest forms of food preservation technology in the world. Indigenous fermented foods such as bread, cheese and wine, have been prepared and consumed for thousands of years and are strongly linked to culture and tradition' (Battcock and Azam-Ali, 1998). The social-economic importance of traditional fermented food common heritage is widely testified by the importance of 'geographical indication' in the worldwide intellectual property law and trade rules. The BIAMFOOD project contributed to enhance the scientific basis for safer traditional wines, cheeses and ciders allowing a better preservation and valorization of this precious socio-economical European 'edible heritage'.

Hobsbawm E. (1983) Introduction: inventing traditions. In The Invention of Tradition, ed. Hobsbawm, T Ranger, pp. 1–14. Cambridge, UK: Cambridge Univ. Press.
Holtzman J.D. (2006) Food and Memory. Annu. Rev. Anthropol. 35: 361–78.
Battcock M., Azam-Ali S. (1998) Fermented Fruits and Vegetables. A Global Perspective. FAO Agricultural Services Bulletin No. 134. Food and Agriculture Organization of the United Nations. Rome.

Overview of impact

The ultimate goal of the present project is the production of fermented foods with no or controlled amounts of biogenic amines, components that form a health risk for consumers. The project has contributed to two main challenges of Theme 2 of the FP7 Cooperation Work Programme **Food, Agriculture and Fisheries, and Biotechnology**: the growing demand for safer, healthier, higher quality food and reducing the risk of food related disorders. Improvements of fermented food products were obtained by focussing on the micro-organisms (Lactic Acid Bacteria; LAB) that are responsible for the production of biogenic amines in the complete food chain from starter to final product. Results were obtained through close collaboration between scientists of many different disciplines and industrial parties resulting in the implementation of scientific results in small-scale food productions. In particular, the project addressed the development of strains and techniques to minimise the human risk due to the production of biogenic amine (BA) by food fermenting LAB, contributed to the quality of food in general and, in particular health aspects of food, and the biomedical implications of the presence of BA and BA-producers in the human gastrointestinal tract. The project bridges scientific fields and spreads excellence to the fermentation industries and the results contribute to the implementation of the EU policy objectives of improving the competitiveness of European industry and enhancing the quality of life of the European citizen.

Safer foods and consumer awareness

The presence of biogenic amines in foodstuffs is an important food safety problem because of the implication of these compounds in food intolerance and intoxication. With every day that passes, the number of people suffering from allergy is increasing. Biogenic amines are not considered to be allergens, but their implication in food intolerance and intoxication are closely related. Biogenic amines do not produce allergic reactions, as physiological reactions to their ingestion are not mediated by the immune system; they rather are toxic compounds that can erroneously be considered as allergic. The symptoms experienced by food allergy and food intolerance (rashes, headaches or feeling bloated) are similar but the mechanism is different. Because of similar symptoms for food allergy, food intolerance and food intoxications, biogenic amine intoxication is often misdiagnosed. For instance, histamine intoxication is often referred to as False Food Allergy or pseudo-allergy. According to the World Health Organisation (WHO), the optimal treatment of allergy consists of the following actions: (i) avoiding the substance that causes the allergy, (ii) undergoing an allergy vaccination programme and (iii) education. The BIAMFOOD project aimed at the first of the described actions, minimizing the content of biogenic amines in fermented foods, which undoubtedly is the most effective way of fighting the symptoms. Therefore, the project will have an important impact on the well-being of a significant part of the European population.

Biogenic amines in foods are introduced at two levels:

- the fermentation process. BA production is an inherent property of the production process. The presence of microbiological flora is essential to the production and is responsible for (part of) the conversion of the substrate into the end-product.
- spoilage. This is a problem general to food and related to shelf-life, food handling, hygienic issues, packaging, etc and has been the subject of several studies, many focusing on the levels of BA in foods.

The BIAMFOOD project deals exclusively with the study of BA formation in foods by microorganisms that inherently are present in the fermentation processes, an aspect that has gained much less attention than food spoilage but will have a much greater impact because it aims at controlling or even eliminating the problem rather than identifying or analyzing the problem. Consequently, the project enhances the consumers trust in the food product itself rather than in the authority that has to safeguard the food products.

The most important biogenic amines in food are histamine, tyramine, putrescine and cadaverine. While histamine has received much attention followed by tyramine due to higher toxicity of these two, there are several reports in the literature concerning other biogenic amines such as putrescine, phenylethylamine and cadaverine which to the least can potentiate the negative effects of the former two. The BIAMFOOD consortium has focussed on the micro-organisms (LAB) that are responsible for the production of the histamine, tyramine, putrescine and cadaverine formed during food fermentation and covered the the complete food chain: from starter to final product. The impact of the capacities and new insights obtained in the project are substantiated by the fermented foods trials performed in collaboration with the food producers (SME's) involved in the BIAMFOOD project.

BA content in food products from different fermentations in different regions and food processing were determined in the project, contributing to consumer awareness of food quality. Histamine levels were found to be higher in cheeses from raw than from pasteurized milk, in processed than in unprocessed cheeses, and in long ripening cheeses. The highest

histamine content was detected in commercial blue cheese samples. Main BA found in cider was putrescine, while they do not seem to contain contain histamine and tyramine. In wines, putrescine and histamine were most frequently encountered and regional differences were observed.

Biomedical impact

One open question addressed in the BIAMFOOD poject was whether BA-producers present in fermented foods and beverages would be able to survive in the gastrointestinal tract (GIT) and still produce BA. A producing organism that comes within the food may, in principle, introduces the capacity to form BA *in situ* even if the food product itself does not contain high BA levels. The BIAMFOOD consortium provided clear evidences on the ability of LAB able to produce BA to survive in the intestinal environment and synthesize BA in the colon. These results offer further evidence of the importance of eliminating/reducing/controlling the presence of BA producing strains in order to manufacture safer foods.

While studies of BA-producing bacteria in relation to human health usually focus on negative aspects of food intolerance and intoxication, the studies in the BIAMFOOD project on the interaction between BA producing bacteria and the human gastrointestinal tract have revealed an anti-inflammatory effect of BA of the tissue lining the gastrointestinal tract, thus helping to prevent the onset of unwanted inflammatory responses such as those taking place in Inflammatory Bowel Disease or Crohn´s disease. BA are shown to be capable of modulating the effector functions of dendritic cells and macrophages, which are critical for maintenance of tissue homeostasis, immunity and resolution of inflammation. Since inflammation is now recognized as a critical component of pathologies such as atherosclerosis, obesity and cancer, our results have implications for human health that might extend beyond the aspects detailed below. In our view, and from a scientific perspective, the following logical step would be testing *in vivo* effects of BA through the use of currently available animal models for inflammation-mediated pathologies.

Macrophages and dendritic cells constitute the essential link between innate and adaptive immunity. Dendritic cells exhibit the unique property of initiating immune responses against foreign microbes, and allow the generation of immune responses appropriately tailored to the pathogen and the surrounding tissue environment. With regard to the latter, macrophages display a huge functional plasticity that allows them to adapt to the surrounding tissue environment and to respond to endogenous and non-self stimuli. In the presence of microbe-derived factors macrophages acquire pro-inflammatory, bactericidal, tumour suppressive and immunogenic activities, in a process commonly referred to as "M1 polarization". Conversely, some cytokines and growth factors variably promote the acquisition of anti-inflammatory, tumour-promoting and pro-angiogenic functions, all of which are grouped under the term "M2 polarization". The sequential occurrence of both opposite polarization states is needed for appropriate resolution of inflammatory processes and adequate tissue repair after injury. Regarding pathology, the shift between macrophage polarization states is particularly relevant in the case of tumours: whereas M1 macrophage-derived pro-inflammatory cytokines contribute to tumour initiation, tumour-derived factors skew macrophage polarization towards the acquisition of an immunosuppressive and tumour-promoting M2 profile which ultimately facilitates tumour metastasis and escape from immune surveillance.

Given the above premises, we have addressed the analysis of the influence that biogenic amines (BA) might have on the functional activities of dendritic cells and macrophages. Our finding that histamine, putrescine, spermine and tyramine promote dendritic cell phenotypic maturation in the absence of a pathogenic stimulus implies that BA can modulate the balance between tolerance and immunity under homeostatic conditions. Moreover, since exposure to BA must coincide with exposure to large concentrations of bacterial derived products, the ability of all tested BA to increase the production of IL-10 by dendritic cells and macrophages exposed to pathogenic stimuli (e.g., LPS), without significantly affecting IL12p40 release, implies that BA preferentially exert an anti-inflammatory activity. This anti-inflammatory action would be particularly relevant in the context of the gastrointestinal tract. Macrophages in the gut are preferentially polarized towards an IL-10^{high} IL-12^{low} profile, as a means to prevent undesired inflammatory responses against the multitude of microbial products in the surrounding tissue. Therefore, the ability of BA to favour IL-10 production might support the anti-inflammatory state of the tissue lining the gastrointestinal tract, thus helping to prevent the onset of unwanted inflammatory responses such as those taking place in Inflammatory Bowel Disease or Crohn´s disease. Consequently, the results derived from our studies suggest that the presence of BA might contribute to diminished immunogenic or inflammatory responses to the bacteria, or bacterial-derived metabolites, contained in traditional food fermentations. This prediction could be evaluated using available animal model of intestinal inflammation and, if verified, might call for a re-evaluation of the effects that BA have been thought to have on human health.

In addition, we addressed the ability of BA to modify the state of macrophage polarization by means of gene expression profiling studies. Our results indicate that macrophages exposed to BA increase the expression of genes (e.g., SERPINB2, THBS1) whose presence correlates with an anti-inflammatory state. Specifically, histamine-exposed macrophages displayed enhanced levels of SERPINB2, whose anti-inflammatory activity has been recently demonstrated. Along the same line, tyramine-exposed macrophages from one donor showed higher levels of THBS1, whose anti-inflammatory nature is well known. The link between inflammation and tumorigenesis is well-established and supported by genetic, pharmacological, and epidemiological data. Thus, whether the macrophage polarizing ability of histamine and tyramine can be extended to other BA deserves further investigations, as it might lead to unanticipated implications, the most important of them being a potential beneficial action of BA in impairing cancer development secondary to chronic inflammation. Conversely, however, BA could also potentially favour progression of ongoing and previously established tumours, as they could re-inforce the establishment of the anti-inflammatory environment required for tumour growth and dissemination.

Industrial relevance

Production of foods and beverages by fermentation is probably the oldest industry in the world with a wide variety of products. This has two important consequences for nowadays fermentation industries: (i) the processes are strongly rooted in traditional, experience-based techniques and (ii) the processes are strongly regionally determined. This has resulted in fragmentation of the process specific technological expertise along food and regional lines as evidenced by the existence of many wine institutes, dairy institutes, wine universities, etc throughout Europe. The BIAMFOOD project counteracts this situation by including three different fermentation products, cheese, wine, and cider from different regions in Europe in the project thereby acknowledging the same biological activities and the same type of

microorganisms (LAB) that underlie the different fermentation processes. Thereby, the project contributes to the awareness in the different industrial branches of a common denominator and cross-semination of knowledge will appear.
The BIAMFOOD project deals exclusively with the study of BA formation in foods by microorganisms that inherently are present in the fermentation processes, an aspect that has gained much less attention than food spoilage but will have a much greater impact because it aims at taking away the problem rather than identifying or analyzing the problem. The goals of the project are to minimizing the formation of BA in the final products rather than detecting BA in the products by spoilage or analyzing their effects on human health. The innovation-driven approach increases the competitiveness of regional food producers and strengthens the local economy in rural regions of Europe which will create employment opportunities, in particular, in smaller industries based in economically weaker parts of Europe.

A database of BA-producing pathways and malate/citrate decarboxylation pathways in LAB was built. The database contains information on the host organism, coding genes, operon structure, operon context, regulatory elements, genetic element, enzyme classification, transporter classification. The database is freely at the disposal to the fermentation industries and all other interested parties to identify and characterize BA-producing LAB in industrial fermentation processes. Ready to use techniques to identify BA-producers and BA-content have been developed and optimized and tailored for use in the final food products and during different stages of the fermentation process. The project has identified regional differences in biogenic amine formation for the same product and differences between different product types. Replacement of BA-producing microbiological activity by other activities may improve the organoleptic properties of the product.

The greatest impact may be expected from the use of added adjunct cultures of BA-degrading LAB. In the project, several strains were isolated capable of degrading histamine, tyramine and/or putrescine. Isolates were from cheese and wine fermentations. In a mini-cheese trial producing Cabrales cheese, co-innoculation of BA-producers and BA-degraders significant reduction of BA content was achieved in the final product as compared to control trials inoculated with only the BA-producer.

Contribution to European regulation and legislation (EFSA)

Specific legislation which includes microbial criteria for BA in foods only covers histamine in fishery products. Criteria for other BAs or other food products do not occur in any national legislation. Commission Regulation EC no. 2073/2005 (as well as the amended EC No. 1441/2007) sets food safety criteria for histamine in two different fishery products. The first is for fish species (particularly within the families *Scombridae, Clupeidae, Engraulidae, Coryfenidae, Pomatomidae,* and *Scombresosidae*) placed on the market during their shelf life and, with a sampling plan 9 units, 2 of the units may be between 100 and 200 mg/kg of histamine, and none above the limit of 200mg/kg. This regulation also applies to fishery products (particulary for the families given above) which have undergone enzyme maturation treatment in brine and placed on the market during their shelf life and, with a sampling plan 9 units, 2 of the units may be between 200 and 400 mg/kg of histamine, and none above the limit of 400mg/kg. For both these products, the analytical method specified in EC no. 2073/2005, is the HPLC method.

The recent increase of biogenic amine content in some fermented foods lead the European Food Safety Authority (EFSA) to enlarge the risk assesment as provided for histamine to other biogenic amines. The EFSA Panel on Biological Hazards (BIOHAZ) set up a working group (WG) to deliver a scientific opinion on risk based control of biogenic amine formation in fermented foods. Members of the BIAMFOOD consortium were also appointed in the WG. The research perfomed by the BIAMFOOD consortium will surely helped EFSA and, in turns, the European Commission in evaluating whether regulation is required in this area of food safety. Especially, histamine has been indicated as the causative agent in several outbreaks of food intoxication. A level of approximately 1000 ppm of total BA in food is believed to elicit toxicity. From a Good Manufacturing Practice (GMP) point of view levels in food of 50 to 100 ppm and 100 to 200 ppm of histamine and tyramine, or a total of 100 to 200 ppm are acceptable. However, it is desirable to reduce these values even further. The development in this project of starter strains and methods for production of cheese, wine and cider as well as of diagnostic tools for the detection of BA-producer micro-organisms has contribute to relieve the problem of BA in the food chain and, at the same time, has increased the awareness of consumers and regulatory agencies like the European Food Safety Authority (ESFA), that may bring to the establishment of limits on the content of BA in fermented foods.

European dimension

The project has brought together researchers from the applied sciences and from fundamental sciences as well as industrial partners from all over Europe. The applied groups study different aspects of BA-producing bacteria in fermentation processes with complementary expertises in specialised fields such as biogenic amine production and malate and citrate fermentation with a link to wine, dairy and cider industries. In the applied sciences where a link exists with a regional industry, research is focussed on one particular fermentation in one particular region. A major impact of the BIAMFOOD project is that it has exceeded this limitation by bringing together research groups linked to specific fermentations in specific regions. This has brought together existing expertises on cider, cheese and wine fermentations residing in Spain (Asturias), France (Normandie, Bordeaux, Bourgogne and Rhone valley) and Italy (Apulia). From a biological point of view these fermentations are similar processes.

Typically, producers of cider, wine and cheese are regional SME’s that are difficult to involve in research projects. The BIAMFOOD project was set up to link a producer to one of the research groups in the consortium with the aim to have direct access to the biological researches and end products. Scientific and technological information generated in the project has been implemented in small-scale production pilots together with the SME’s. This has resulted in a intensive and lasting interaction between research group and the SME’s.

During the project intensive collaboration have been set up between the partners of the project resulting in many joined publications in scientific journals. During the 3 years of the project, 40 papers were published in peer reviewed journals which by itself has put the study of biogenic amines in relation to food fermentations in focus of the scientific community. Twelve out of the 40 papers were joined publications of the partners of the BIAMFOOD consortium. One paper was published involving authors of all beneficiaries. BIAMFOOD clearly has increased academic-industrial collaboration within the European community and thereby has promoted the competitiveness of the European industrial base.

Finally, the European nature of this project has established a network between the younger scientists (post-graduates and post-doctoral researchers) who met at the biannual project meetings of the consortium. A PhD student of the University of Rosario (beneficiary IBR), Argentina has worked for three months at the University of Groningen (RUG), The Netherlands on a joined project and a PhD student of the University of Foggia (UNFG), Italy has worked during the second year of the project at the Centro Investigaciones Biológicas (CIB), Spain, also on a joined project. This will contribute to future collaborations and will increase the strength of European research endeavours.

Dissemination of foreground

The composition of the BIAMFOOD consortium is optimized to disseminate the results of the project to the industries. One major industrial beneficiary CH (Christian Hansen A/S) and six specialized fermentation product producing industries in the project maximize the potential commercial utilisation of the results. The participation of two SMEs and one research institute who's main mission it is to support local industries not only provides access to the production facilities and materials, but also warrants the flow of information back to the technology end-users. Beneficiary IR, Inter Rhône is an SME promoting AOC wines of the Rhône Valley, France. Beneficiary ADRIA Normandie is a technical centre in Normandie, France that is in contact with SMEs as well as bigger companies and has a focus on safety of fermented products, mainly cider and cheese. Finally, beneficiary IPLA is an institute located in Asturias, Spain and focuses on the production of traditional cheeses and cider. The participation of 4 SMEs and 2 large enterprises that are producers of fermentation products warrant the immediate implementation of the findings of the project into the fermentations processes when applicable.

World Wide Web

The BIAMFOOD website (http://www.biamfood.eu) has been officially presented on the first workshop organized by the project in Bordeaux, October 3 and 4, 2008. The website provides a link to the public database of the BIAMFOOD consortium: BIAMBASE which contains a collection of BA producing pathways in microorganisms and the results of the screening of the collections of the beneficiaries for BA-producing organisms.

The FP6 project EuroFIR (http://www.eurofir.net/) and MoniQa (http://www.moniqa.org/) were contacted (Paul Finglas/Dawn Wright and Daniel Spichtinger for EuroFir and MoniQa, respectively) for mutual use of dissemination channels which has resulted in links to the different projects on the project web sites.

Funding of the BIAMFOOD project was announced by interviews and press releases on the Chr. Hansen (CH) web site ('Chr. Hansen gets research funds from the European Commission', October 2008 on http://www.chr-hansen.com).

The project was announced at the web sites of wine producer Cantine "D'Alfonso del Sordo" S.R.L., Italy (beneficiary SORDO; http://www.dalfonsodelsordo.it) and promoted through local television interviews and at the website of the University of Foggia, Italy ((http//:www.unifg.it).

Presentations to professionals and policy makers

The BIAMFOOD project was presented prominently with three posters and a power point presentation by beneficiary's UNFG/SORDO at the Innovation Festival which took place at 3-5.12.2008 in Bari, Italy. At the festival the project was discussed with Antonio di Giulio, Head of Unit E3: Food – Health – Well-being - Directorate E: Biotechnologies, Agriculture and Food and with the South Australian Government Commitee and the General Manager - Business Development of the he Australian Wine Research Institute (AWRI). The latter resulted in a scientific collaboration between Eveline Bartowsky, Senior Research Microbiologist, The Australian Wine Research Institute, Adelaide, Australia and the University of Foggia, Italy.

BIAMFOOD Workshop Foggia. A workshop 'Controlling biogenic amines in fermented foods' was organized in Foggia, Italy on 12th of December 2009. The workshop was widely advertised throughout Italy through a brochure and on the internet, e.g. http://www.teleradioerre.it/news/articolo.asp?idart=49170. The workshop was visited by several politicians, and representatives of wineries, cheese and pasta producers.

The 2° International Symposium "Wine Microbiology and Safety: from the vineyard to the bottle" (Microsafetywine)", Martina Franca (Taranto, Italy), November 19-20 2009 has dedicated an entire session (Session 4) on Biogenic ammines in wine. Beneficiaries G. Spano (UNFG) and B. Bach (IR) have given oral presentation promoting the BIAMFOOD project.

Further presentations of the BIAMFOOD project were presented by beneficiaries to the Italian National Research Council (CNR), Italy, the TOP Institute Food and Nutrition, The Netherlands, Chambre d'Agriculture de la Gironde, France, Vinitech Bordeaux, France, International exhibition for the vine/wine and fruit/vegetables sectors, France, Spanish Society of Rheumatology, Spain, 3rd International Symposium "Macrowine", Italy and the Center of Functional Genomic, Fiorenzuola d'Arda, Italy.

Papers were published in professional journals or presented at professional meetings to promote the BIAMFOOD project among professionals: Foodnavigator, Union Girondine des vins de Bordeaux, Avenir Agricole et Viticole Aquitain, Le Vigneron des Côtes du Rhône et du Sud Est (2x), National Conference of Italian Association of Agricultural Science Societies (AISSA), Connexions (www.connexions-normandie.fr).

Interaction with related FP projects

EUROFIR (European Food Information Resource Network). The coordinator of the FP6 project EuroFIR was contacted for the exchange of information on methodologies and the use of their established dissemination channels to bring foreground of the BIAMFOOD project under the attention of relevant industrial and societal parties. Extensive email discussions with coordinator Paul Finglas on mutual use of dissimination channels has resulted in links to both projects on the web sites. In addition, BIAMFOOD was invited to present the project at the 3rd International EuroFIR Congress in Vienna, Austria to be held from 8-10 September 2009. Guisseppe Spano of the University of Foggia (UNFG) has presented the BIAMFOOD project in an oral presentation. In addition, a review entitled **'Risk assessment of biogenic amines in fermented food'** was published in special issue of the Eurpean Journal of Clinical Research dedicated to the 3rd International EuroFIR Congress in Vienna, Austria. The full reference:
G. Spano, P. Russo, A. Lonvaud-Funel, P. Lucas, H. Alexandre, C. Grandvalet, E. Coton, M. Coton, L. Barnavon, B. Bach, F. Rattray, A. Bunte, C. Magni, V. Ladero, M. Alvarez, M.

Fernandez, P. Lopez, P. F. de Palencia, A. Corbi, H. Trip, and J. S. Lolkema (2010) Biogenic amines in fermented foods. *Eur. J. Clin. Nutr. 64,* Suppl 3:S95-100.

MoniQa (Monitoring and Quality Assurance in the Food Supply Chain). Coordinator Daniel Spichtinger of the FP7 Network of Excellence MoniQa was contacted for mutual use of dissimination channels which has resulted in links to both projects on their web sites. A representative organization of the BIAMFOOD consortium was invited to become an associate member of MoniQa. The University of Foggia was accepted as associate member in this capacity.

Masterclass

A masterclass 'Transport Processes in Industrial Microorganisms' was organized in Groningen, the Netherlands from 22-26 of March 2010. The workshop was organized by the Groningen Biomolecular Sciences and Biotechnology Institute in collaboration with the BIAMFOOD project and B-Basic, a Dutch initiative on 'Biobased sustainable industrial chemistry'. BIAMFOOD masters were Patrick Lucas of the University Bordeaux Victor Segalen, Bordeaux (beneficiary UB2), Fergal Rattray of Christian Hansen, Denmark (beneficiary CH) and Juke Lolkema of the University of Goningen, The Netherlands. The full program is available at http://www.rug.nl/gbb/studyatgbb/masterclasses/tpim/index. In addition to participants from several BIAMFOOD beneficiaries, representatives from leading Dutch industries attended the meeting. Hein Trip (RUG) and Andrea Romano (UB2) presented there work in the BIAMFOOD project in oral presentations.

EFSA (European Food Safety Authority) working group

A request for a scientific opinion regarding a "Self-tasking mandate on risk based control of biogenic amine formation in fermented foods (EFSA-Q-2009-00829)" was proposed by EFSA to the Panel of Biological Hazards (BIOHAZ) and has been accepted. A working group was set up for this mandate. Giuseppe Spano of the University of Foggia (partner UNFG) has become a member of this working group as a representative of the BIAMFOOD consortium.

Methods and patents

A Spanish patent application has been filed by partner CIB entitled 'Vectores de fusión transcripcional para regiones promotoras uni- y bidireccionales para su uso en bacterias lácticas'.

A new method was developed by partner IR involving a reversed-phase separation by HPLC and UV-vis detection of the aminoenones for the simultaneous analysis of biogenic amines, amino acids, and ammonium ions in wine and beer. This method has been proposed as a reference method to the International Organization of Vine and Wine (March 2010).

Partner UB2 has developed a method based on thin layer chromatography for determining BA in supernatants of bacterial cultures. This task was not initially scheduled in BIAMFOOD. In October 2010, UB2 and SARCO have initiated a one-year project funded by CIVB (Interprofessional Council of Bordeaux Wines) with the aim to optimize the method for determining BA in wine.

Oral and poster presentations at scientific meetings

Members of the BIAMFOOD consortium have presented their work in oral presentations at the Food Micro 2008 Conference, Aberdeen, Scotland, the Joint CSIC-CRUSA meeting, San José, Costa Rica, Seminar at CINVESTAV, México DF, Mexico, 5th International meeting on Biotechnology, Spain, 46th Annual Meeting Argentine Society for Biochemistry and Molecular Biology. A total of 67 posters showing the results of the BIAMFOOD project were presented at international and national meeting throughout the world.

Papers in peer reviewed scientific journals

The project has yielded 40 papers in peer reviewed scientific journal. Another 9 papers have been submitted for publication. These are:

*1. Russo, P., Fernández de Palencia, P., Romano, A., Lucas, P., Spano, G. and López, P. (2011) Biogenic amine production enhances the survival of *Lactobacillus brevis* IOEB 9809 through the gastrointestinal tract. submitted .

*2. Arena M.P., Romano, A., Capozzi, V., Russo, P., Beneduce, L., Grieco, F., Lucas P. and G. Spano (2011). Expression of *Lactobacillus brevis* IOEB 9809 tyrosine decarboxylase and agmatine deiminase genes in wine. J. of Appl. Microbiol., submitted.

3. Tristezza, M., Vetrano, C. Bleve, G., Grieco, F., Tufariello, M., Mita, G. Spano, G. and F. Grieco (2011) The selection of autochthonous yeast and preparation of starter cultures for the industrial production of wines from cv. Negroamaro. J. Microbiol. and Biotechnol., submitted.

4. Repizo G., Mortera P and Ch. Magni (2011). Disruption of the alsSD operon of *Enterococcus faecalis* impairs the growth on pyruvate at low pH. Microbiol. submitted

5. Suárez, CA, Blancato, VS and Magni, C. (2011). CcpA represses the expression of the divergent cit operon of *Enterococcus faecalis* through multiple cre sites. J Bacteriol submitted

6. Espariz, M, Repizo, G, Blancato, V, Mortera, P, Alarcón, S and Magni, Ch (2011) Characterization of Two Paralogous Genes Encoding a Malic Enzyme and a Soluble Oxaloacetate Decarboxylase in *Enterococcus faecalis*. FEBS Journal submitted

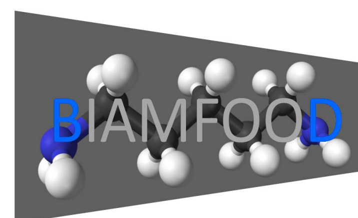
7. C.A. Suárez, V.S. Blancato and Ch. Magni (2011) CcpA represses the expression of the divergent cit operon of Enterococcus faecalis through multiple *cre* sites. J. Bacteriol, submitted

8. Pudlik, A. and Lolkema, J.S. (2011) Mechanism of citrate metabolism by an oxaloacetate decarboxylase mutant of *Lactococcus lactis* IL1403. J. Bacteriol. submitted.

9. Trip, H., Mulder, N. and Lolkema, J.S. (2011) Heterologous expression of the histidine decarboxylation pathway of *Streptococcus thermophilus* CHCC1524 in *Lactococcus lactis* confers acid stress resistance. J. Bacteriol. submitted.

Project details

BIAMFOOD



Controlling Biogenic Amines in Traditional Food Fermentations in Regional Europe

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website: http://www.biamfood.eu
Project web site: www.biamfood.eu

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