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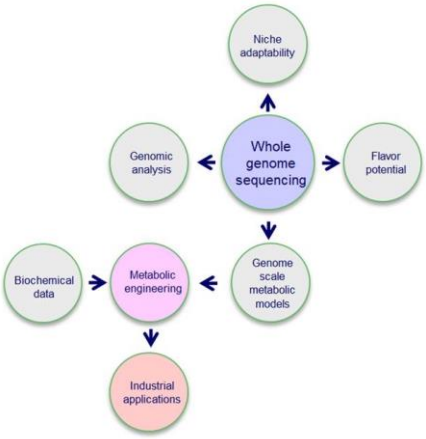
# *Lactobacillus paracasei*: Genomics, Metabolomics and Applications




## Key external stakeholders:

- Commercial culture suppliers
- Fermented dairy food producers
- Flavour ingredient producers
- Wider dairy industry
- Culture and flavour research communities

## Practical implications for stakeholders:

The study discovered that strains from the *Lactobacillus casei* group are diverse in terms of their metabolic potential and their ability to diversify flavour, particularly in applications such short-aged Cheddar cheese. The cultures studied in this project are a potential resource for companies interested in flavour diversification of their product portfolio.

## Main results:

- The *Lactobacillus paracasei* species is characterised by genetic and metabolic diversity, supporting their potential for variability in volatile production.
- The increase in the complexity of environment minimises the phenotypic variation observed.
- Genome sequencing confirms the high level of diversity between *L. paracasei* strains from the same niche.
- Strains have the potential for cheese flavour enhancement, especially in short ripened cheeses.

## Opportunity / Benefit:

An in-depth knowledge of the metabolic potential of starter strains and the key technological properties which make their application in the industry possible, such as flavour and texture can allow starter blends to be 'tailor made' to suit industry needs. This approach also allows for the potential improvement of these and other key characteristics in existing strains, strains which are at the core of the dairy industry. Applying this knowledge to starter culture development is enabling the generation of superior starters and novel products for future market expansion.

## Collaborating Institutions:

(INRA), University College Cork (UCC).



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## 1. Project background:

The genus Lactobacillus consists of more than 200 species and subspecies present in various environments such as plants, fermented food products (dairy, meat, wine), and both the human and animal gastrointestinal and reproductive tracts. One of the most studied groups of this genus is the *Lactobacillus casei* group, which includes the species *Lactobacillus casei*, *Lactobacillus paracasei*, and *Lactobacillus rhamnosus*. Strains of this group show remarkable ecological adaptability and have been isolated from all the typical habitats of lactobacilli. Such a diverse range of sources facilitated a broad spectrum of applications of strains of this species in dairy production (adjunct cultures), and in health-related (probiotics, bacteriocins) and biotechnological fields. These characteristics and potential applications make the species of the *L. casei* group one of the best explored within the *Lactobacillus* genus. While certain strains of these species are perhaps best known for their characteristic health benefits, other strains of *Lb. casei* and *Lb. paracasei* are commonly found as the dominant species of nonstarter lactic acid bacteria (NSLAB) in ripening cheese and are likely to play a role in the development of flavour in these products. The development of flavour results from a complex network of metabolic reactions, which includes three main processes: sugar metabolism (glycolysis), lipid degradation (lipolysis) and protein catabolism (proteolysis). Although sugars, mainly lactose, and lipids can be metabolized to flavour compounds, the proteolytic process is seen as particularly important for flavour development. The aim of this study was to compare the genomic and metabolic characteristics of strains of the *L. paracasei* group and to assess their suitability for flavour production in dairy applications.

## 2. Questions addressed by the project:

- What is the extent of the genetic and metabolic diversity within the *Lactobacillus casei* group?
- What is the potential of strains from this group for the production of flavor compounds?
- Does the model system used for flavor compound analysis influence the volatile profile of a given strain?
- Does the extraction method used for flavor compound analysis influence the volatile profile of a given strain?
- What are the potential applications of strains of the *Lactobacillus casei* group in dairy processing?

## 3. The experimental studies:

- **Genetic, enzymatic and metabolite profiling.** Following confirmation of species by 16S rRNA PCR, the diversity of the isolates was determined by pulsed-field gel electrophoresis. The activities of enzymes involved in the proteolytic cascade were assessed and significant differences among the strains were observed. Ten strains were chosen based on the results of their enzymes activities and they were analysed for their ability to produce volatiles in media with increased concentrations of a representative aromatic, branched chain and sulphur amino acid. Volatiles were assessed using gas chromatography coupled with mass spectrometry.
- **Monitoring production of volatile compounds in model systems.** The capabilities of strains of *L. paracasei* to produce volatile compounds in two model systems were assessed. Model system 1



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(MS1) was based on a synthetic amino acid mix, while model system 2 (MS2) was based on processed cheese curd. Subsequently, the strain diversity was mapped using a chemometric approach, which showed different abilities of strains for volatile production in the 2 model systems used.

- **Determination of the potential effect of the extraction method used on volatile profiles.** Two extraction techniques were assessed. In method A, Solid-Phase Microextraction (SPME) was performed using CarboxenTM/ divinylbenzene/ polydimethylsiloxane fibre. In Method B, HeadSpace-Trap (HS-Trap) was performed with Tenax™ trap. After extraction, GC was run with DB- and StabilWaxTM columns in methods A and B, respectively. Obtained chromatograms were processed by XCMS package of statistical software R. Analysis of variance (ANOVA), least significant difference test (LSD) and Principal Component Analysis (PCA) were performed in R.

- **Comparative genomic and metabolic analysis of *Lactobacillus paracasei* cheese isolates**. We compared the genome sequences of three *L. paracasei* strains isolated from mature Cheddar cheeses, two of which (DPC4206 and DPC4536) shared the same genomic fingerprint by PFGE, but demonstrated varying metabolic capabilities.

- **Cheese production.** Cheeses were manufactured at pilot scale, i.e., 500 L vats, at Moorepark Technology Ltd (Fermoy, Cork, Ireland).

## 4. Main results:

- **Genetic, enzymatic and metabolite profiling of the *Lactobacillus casei* group reveals strain biodiversity and potential applications for flavour diversification.**
280 strains from the DPC culture collection at Teagasc Moorepark have been analysed and subsequent phylogenetic analysis confirmed 90 diverse strains of the *Lb. casei/paracasei* group. The observed level of genetic diversity can be considered as very broad, since the majority of isolates have the same origin of isolation. The analysed strains, including two strains with an identical genetic fingerprint, showed variable phenotypic traits, as observed in assays determining the activities of proteolytic cascade enzymes. Additionally, the strains demonstrated different capacities for production of flavour compounds from amino acids, and two strains, DPC2068 and DPC4206, were particularly diverse in their volatile production. It can be inferred that strains of the *L. casei* group have different abilities for volatile production, which makes them potentially useful for dairy product flavour diversification.

- **The choice of model system and analytical approach influences volatile flavor profiles.**
In order to identify strains of *L. paracasei* with the most diverse volatile profiles, ten genetically distinct strains were tested in two model systems. Model system 1 (MS1) was based on a synthetic amino acid mix, while model system 2 (MS2) was based on processed cheese curd. In MS1, the tested strains produced volatiles including acids, alcohols, esters, aldehydes and ketones, and three strains (DPC2071, DPC3990 and DPC4206) demonstrated the most variable volatile production. When tested in MS2, strains produced acids, alcohols, esters and ketones, and metabolised aldehydes. However, there was much less variation in the volatile profiles of strains compared to those observed in MS1, and the only strain that was considered as slightly different was DPC4206. Additionally, to determine the potential effect of the extraction method used on volatile profiles, two extraction techniques HeadSpace Trap (HS-Trap) and HeadSpace Solid Phase Microextraction (HS-SPME) were compared. Analysis of MS1 samples demonstrated that HS-SPME preferably extracted alcohols, esters and acids, while HS-Trap showed higher selectivity towards short aldehydes and ketones, pyrazine derivatives and sulphur-containing compounds. When the diversity of strains was analysed based on these results, the same three strains were outlined as the most different. Although differences in volatile compound extraction exist, the choice of extraction method has a little impact on the screening of the metabolic diversity of set of strains.

- **Comparative genome analysis of three cheese isolates of *Lactobacillus paracasei* demonstrates considerable genomic diversity in strains of the same origin.**
We elucidated and compared the genome sequences of three strains isolated from mature Cheddar



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cheese, two of which (DPC4206 and DPC4536) had the same genomic fingerprint by PFGE, but demonstrated varying metabolic capabilities. Genome sizes varied from 2.9 Mbp for DPC2071, to 3.09 Mbp for DPC4206 and 3.08 Mpb for DPC4536. Strain DPC2071 possessed an unusually high number of plasmids (11), while strain DPC4206 had one plasmid, and DPC4536 carried no plasmids. Strains DPC4206 and DPC4536 possessed higher numbers of carbohydrate metabolism genes compared to DPC2071, and that this was confirmed in a set series of sugar fermentation of experiments, where DPC2071 was not able to utilise maltose, inulin, tagatose and sorbose as energy sources. Interestingly, strain DPC4536 did not possess the gene encoding 6-phospho beta galactosidase, the initial enzyme in lactose metabolism. Strains also showed variability in the presence of phage content remnants and phage protection systems. While a type I CRISPR system was detected in DPC2071, a type II CRISPR was present in DPC4206 and DPC4536, but different spacers were identified in the latter two strains. Regarding flavour forming capacities, all three strains had very similar genetic content of relating to enzymes of the proteolytic cascade genes. However, differences in activities of enzymes that contribute to cheese flavour development were confirmed both in in vitro conditions and in cheese trials, where fully ripened cheeses developed different flavours, confirming the importance of expression regulatory mechanisms. The findings confirm a considerable level of heterogeneity within the *L. paracasei* species, even within strains of the same origin.

- **Certain *Lactobacillus paracasei* strains have the potential to act as flavor adjuncts in short-aged Cheddar cheese.**
  Three strains of *L. paracasei* (DPC2071, DPC4206, and DPC4536) were evaluated for their contribution to the enhancement and diversification of flavor in short-aged Cheddar cheese. The strains were selected based on their previously determined genomic diversity, variability in proteolytic enzyme activities and metabolic capability in cheese model systems. The addition of adjunct cultures did not affect the gross composition or levels of lipolysis of the cheeses. The levels of free amino acids (FAA) in cheeses showed a significant increase after 28 days of ripening. However, the concentrations of individual amino acids in the cheeses did not significantly differ except for some amino acids (aspartic acid, threonine, serine, and tryptophan) at Day 14. Volatile profile analysis revealed that the main compounds that differentiated the cheeses were of lipid origin, such as long chain aldehydes, acids, ketones, and lactones. This study demonstrated that the adjunct *L. paracasei* strains contributed to the development and diversification of compounds related to flavor in short-aged Cheddar cheeses.

## 5. Opportunity/Benefit:

The abolition of milk quotas in 2015 resulted in a major expansion in milk production in Ireland (Food Harvest 2020 report). Cheese was targeted as a strategic end product to utilise a significant proportion of this extra milk. Expansion has occurred both in terms of volume and also in increased cheese diversification. Research in cheese diversification at Teagasc is currently focusing on manipulation of specific cheese physico-chemical, biochemical, and microbiological parameters. The performance of various starter and adjunct cultures is being studied under varying processing conditions to underpin development of a range of cheeses with diverse characteristics, flavours and functionalities which are capable of being made on commercial Irish cheese manufacturing plants. In addition, a recent development has seen an evolving relationship between TFRC and the Irish Dairy Board focusing on the development of a pipeline of new and diverse cheese types with specific flavours and functionalities to target key market opportunities. Cheese starters/adjuncts are a primary driver of flavour development, and thus cultures are a critical component in the national diversification strategy in cheese.

## Dissemination:

The work outlined in this project has been disseminated by means of peer-reviewed publications (see below) and has been presented at a number of conferences, both national and international, including the American Dairy Science Association Annual Meeting 2018, Knoxville, TN, USA; the 12th International Symposium on Lactic Acid Bacteria, the Netherlands, 2017; the IDF International Symposium on Cheese Science and Technology, Dublin, 2016; the 44th Annual Food Research Conference, Cork, 2015, and the 9th Cheese Symposium, Cork, 2014.



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**Main publications:**

Peer-reviewed publications:


- McAuliffe O, Kilcawley K, Stefanovic E. 2018. Genomic investigations of flavor formation by dairy microbiota. J Dairy Sci. 2018 Oct 18. pii: S0022-0302(18)30989-5. doi: 10.3168/jds.2018-15385. [Epub ahead of print]
- Stefanovic E, McAuliffe O. 2018. Comparative genomic and metabolic analysis of three *Lactobacillus paracasei* cheese isolates reveals considerable genomic differences in strains from the same niche. BMC Genomics. 2018 Mar 20;19(1):205. doi: 10.1186/s12864-018-4586-0.
- Stefanovic E, Kilcawley KN, Roces C, Rea MC, O'Sullivan M, Sheehan JJ, McAuliffe O. 2018. Evaluation of the potential of *Lactobacillus paracasei* adjuncts for flavor compounds development and diversification in short-aged Cheddar cheese. Front Microbiol. 2018 Jul 5;9:1506. doi: 10.3389/fmicb.2018.01506.
- Stefanovic E, Fitzgerald GF, McAuliffe O. 2017. Advances in the genomics and metabolomics of dairy lactobacilli. Food Microbiol. 2017 Feb;61:33-49. doi: 10.1016/j.fm.2016.08.009.
- Stefanovic E, Kilcawley KN, Rea MC, Fitzgerald GF, McAuliffe O. 2017. Genetic, enzymatic and metabolite profiling of the *Lactobacillus casei* group reveals strain biodiversity and potential applications for flavour diversification. J Appl Microbiol. 2017 Feb 15. doi: 10.1111/jam.13420.
- Stefanovic E, Fitzgerald G, McAuliffe O. 2017. Draft genome sequences of three *Lactobacillus paracasei* strains, members of the nonstarter microbiota of mature Cheddar cheese. Genome Announc. 2017 Jul 20;5(29). pii: e00655-17. doi: 10.1128/genomeA.00655-17.
- Stefanovic E, Thierry A, Maillard MB, Bertuzzi A, Rea MC, Fitzgerald G, McAuliffe O, Kilcawley KN. 2017. Strains of the *Lactobacillus casei* group show diverse abilities for the production of flavor compounds in 2 model systems. J Dairy Sci. 2017 Sep;100(9):6918-6929. doi: 10.3168/jds.2016-12408. Epub 2017 Jul 12.
- Stefanovic E, Casey A, Cotter P, Cavanagh D, Fitzgerald G, McAuliffe O. 2016. Draft genome sequence of *Lactobacillus casei* DPC6800, an isolate with the potential to diversify flavor in cheese. Genome Announc. 2016 Mar 3;4(2). pii: e00063-16. doi: 10.1128/genomeA.00063-16.


Book chapters:


- Stefanovic E, McAuliffe O. 2018. A genomic perspective on niche adaptability in *Lactobacillus*. In *Lactobacillus* Genomics and Metabolic Engineering. Ed. S. M. Ruzal. Caister Academic Press, Norfolk, UK.
- McAuliffe, O. 2017. Genetics of Lactic Acid Bacteria. *In* CHEESE: CHEMISTRY, PHYSICS AND MICROBIOLOGY, FOURTH EDITION. Eds. P.F. Fox, P.L.H. McSweeney, P. Cotter and D. W. Everett. Elsevier, The Netherlands.


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