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Diversité des bactéries halophiles dans l'écosystème fromager et étude de leurs impacts fonctionnels

Caroline Kothe

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Diversité des bactéries halophiles dans
l'écosystème fromager et étude de leurs
impacts fonctionnels

*Diversity of halophilic bacteria in the cheese ecosystem and
the study of their functional impacts*

Thèse de doctorat de l'université Paris-Saclay

École doctorale n° 581 Agriculture, Alimentation, Biologie, Environnement
et Santé (ABIES)

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**Thèse présentée et soutenue à Paris-Saclay,
le 01/04/2021 par**

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“A master in the art of living draws no sharp distinction between her work and her play; her labor and her leisure; her mind and her body; her education and her recreation. She hardly knows which is which. She simply pursues her vision of excellence through whatever she is doing, and leaves others to determine whether she is working or playing. To herself, she always appears to be doing both.”

Adapted to Lawrence Pearsall Jacks

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Liste de publications

Publications associés au projet de thèse dans des revues scientifiques :

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Kothe, C. I.; Renault, P. Metagenomic driven characterization of halophilic bacteria in cheese rinds (to be submitted)

Kothe, C. I.; Monnet, C.; Irlinger, F.; Renault, P. *Halomonas citridevorans* sp. nov., *Halomonas minus* sp. nov., *Halomonas casei* sp. nov. and *Halomonas flavum* sp. nov., four novel species isolated from French cheese rinds (under review in International Journal of Systematic and Evolutionary Microbiology).

Kothe, C. I.; Monnet, C.; Irlinger, F.; Renault, P. *Psychrobacter casei* sp. nov. and *Psychrobacter translucens* sp. nov., two new species isolated from French cheese rinds (under review in International Journal of Systematic and Evolutionary Microbiology).

Kothe, C. I.; Monnet, C.; Landaud, S.; Renault, P. Functional properties of food and environmental halophilic Gammaproteobacteria strains on cheese-agar models (to be submitted)

Kothe, C. I.; Delbarre-Ladrat, C.; Renault, P.; Passerini, D. (2020). Draft-genome sequence data and phylogenomic comparison of two marine-sourced bacterial strains *Pseudoalteromonas* sp. MIP2626 and *Psychrobacter* sp. BI730. Data in Brief, 31, 105898. <https://doi.org/10.1016/j.dib.2020.105898>

Kothe, C. I.; Renault, P. Meta-analysis of five traditional Brazilian cheese varieties (to be submitted)

Communications lors de congrès :

Kothe, C. I.; Dridi, B.; Renault, P. (2019). Origin and evolution of halophilic bacteria: from environment to cheese. 5th International Conference on microbial diversity, September 25-27, Italy (oral communication).

Liste des abréviations

ADN: Acide désoxyribonucléique

ANI: Average Nucleotide Identity

ARN: Acide ribonucléique

a_w: Activité de l'eau

BLAST: *Basic Local Alignment Search Tool*

GRAS: *Generally Recognized As Safe*

GYPB: *Glucose, Yeast extract, Peptone and Beef extract*

GC-MS: *Gas chromatography–mass spectrometry*

EFFCA: *European Food and Feed Cultures Association*

EFSA: *European Food Safety Authority*

KCl: Chlorure de potassium

IDF: *International Dairy Federation*

ISO: *International Organization for Standardization*

LAB: *Lactic Acid Bacteria*

MB: *Marine Broth*

MAG: *Metagenome-Assembled-Genome*

MGM: *Modified Growth Medium*

MFCs: *Microbial Food Cultures*

MGL: *Methionine gamma-lyase*

NaCl: Chlorure de sodium

NCBI: *National Center for Biotechnology Information*

NSLAB: *Non-Starter Lactic Acid Bacteria*

PCR: *Polymerase Chain Reaction*

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CONTEXTE DU PROJET

Encadré 1 : Synopsis

Ce Projet vise à explorer les bactéries halophiles présentes, et parfois abondantes, dans les croûtes de fromage. Ainsi, étant donné la faible quantité d'isolats disponibles dans des centres de ressources microbiennes, nationaux et internationaux, pour ce groupe de bactéries dans l'alimentation, nous avons utilisé des stratégies pour isoler une diversité d'espèces représentantes des surfaces de fromage. Ces espèces ont ensuite été caractérisées par la génomique et la métagénomique. Enfin, l'impact technologique des souches halophiles environnementales et alimentaires a été évalué dans un modèle de fromage synthétique.

Les communautés microbiennes sont considérées comme l'un des principaux déterminants de la diversité et de la qualité du fromage [1]. Ces écosystèmes sont composés d'une grande variété de microorganismes (tels que des levures, des moisissures et des bactéries), qui peuvent être ajoutés par l'homme, selon la technologie ou les caractéristiques souhaitées, ou qui peuvent s'insérer de manière fortuite tout au long de la chaîne de production. Au cours des dernières années, les progrès de la biologie moléculaire ainsi que l'utilisation de la technologie de séquençage de nouvelle génération ont permis d'établir une image de plus en plus précise de la biodiversité des communautés microbiennes dans les aliments et de leur évolution au cours des étapes de production [2]. En particulier, pour les croûtes de fromages affinés, des études récentes basées sur des méthodes indépendantes des cultures (comme la métagénomique) ont montré la dominance de certains genres halophiles tels que *Halomonas*, *Pseudoalteromonas* et *Psychrobacter* [3, 4]. Ces bactéries sont généralement rencontrées dans des environnements naturels salés, comme les lacs, les mers et les sols. Bien que les halophiles soient aussi occasionnellement isolés des aliments salés, comme les produits de la mer, le sauce de soja et les croûtes de fromages, il existait, au début de ce Projet, une carence en espèces alimentaires disponibles dans les cultures de collections publiques pour des études physiologiques.

Dans ce contexte, les objectifs de ce Projet de thèse étaient d'isoler et caractériser des représentants halophiles dominants dans les croûtes de fromage et étudier leurs effets potentiels technologiques. Les axes de recherche ont été explorés pour les thèmes suivants :

- Isolement systématique des souches fromagères avec des milieux de culture à base de sel et leur distribution dans les métagénomomes de croûtes de fromage;
- Stratégies ciblées pour acquérir des représentants halophiles, faiblement cultivés, en utilisant les données de la métagénomique *shotgun*;
- Caractérisation taxonomique des souches isolées et proposition de nouvelles espèces;
- Impact technologique de souches halophiles alimentaires et environnementales des genres *Halomonas*, *Pseudoalteromonas* et *Psychrobacter* dans un modèle fromage-agar.

Ce manuscrit est composé de trois chapitres. Le premier chapitre est une introduction générale divisée en trois parties. La première décrit la classification des fromages et la diversité des microorganismes présents dans cet aliment. La deuxième partie concerne le sel et ses fonctions potentielles dans les aliments, en plus de l'exploiter comme une source probable de sélection de microorganismes. La troisième catégorise les principaux genres halophiles dans les écosystèmes naturels et alimentaires, en approfondissant la connaissance des espèces présentes dans les fromages et leurs rôles potentiels.

Le deuxième chapitre présente les résultats obtenus au cours de ce Projet et comporte quatre parties, qui correspondent aux axes de recherche développés suivantes: (i) utilisation de méthodes de culture, de la génomique et de la métagénomique pour révéler les halophiles et les halotolérants dans les fromages; (ii) application de stratégies en associant les données de la métagénomique et de la culture pour isoler les microorganismes faiblement cultivés; (iii) proposition de nouvelles espèces halophiles; (iv) impact technologique des souches environnementales et alimentaires dans un modèle fromage-agar. Ces résultats sont présentés sous la forme d'articles scientifiques, rédigés en anglais, et accompagnés d'une introduction et d'une conclusion, en français. Dans le dernier chapitre les principaux résultats sont récapitulés et discutés de manière générale, en proposant de nouvelles perspectives pour ce thème de recherche.

En outre, deux autres articles réalisés pendant mon travail de thèse sont présentés en Annexes. Le premier est un article de données, réalisé en collaboration avec l'Ifremer (Institut français de recherche pour l'exploitation de la mer), dans lequel ont été effectuées des analyses génomiques sur deux génomes marins halophiles. Le deuxième article concerne l'étude des fromages brésiliens traditionnels, dont la composition taxonomique a été exploré par des approches métagénomiques.

CHAPITRE I – INTRODUCTION BIBLIOGRAPHIQUE

1. Le fromage: un écosystème à découvrir

Encadré 2 : Sources de microorganismes dans les fromages

Au-delà des ferments ajoutés au fromage pour des caractéristiques technologiques spécifiques, l'environnement fromager héberge une variété de microorganismes provenant de diverses sources (caves d'affinage, équipements, ingrédients, etc). En effet, Wolfe *et al.* (2014) ont estimé que 60% des genres bactériens et 25% des genres fongiques détectés dans les croûtes de fromages (n=137) proviennent de sources externes. Une grande partie de cette flore adventice correspond à des bactéries peu étudiées, comme les *Halomonas*, *Pseudoalteromonas*, *Psychrobacter* et *Vibrio*, genres qui sont généralement associés aux écosystèmes naturels comme l'eau de mer. Ainsi, une des sources probables de la sélection de cette communauté dans les aliments est le sel marin, ingrédient utilisé lors de la fabrication du fromage.

Les fromages comptent parmi les aliments les plus anciens fabriqués par les êtres humains. Ils ont été produits et consommés pendant des milliers d'années et leurs processus de fabrication sont adaptés à l'évolution des technologies, des contextes sociaux et économiques dans diverses parties du monde [5]. Une large gamme de fromages est transformée à partir d'une seule matière première - le lait - entraînant la croissance de bactéries, de levures et de moisissures qui jouent un rôle essentiel dans le développement de ce produit [1]. Ainsi, la production de fromage est inextricablement liée à la microbiologie, ce qui rend son histoire et la vaste science du fromage et des microorganismes particulièrement uniques.

Actuellement, il existe plus de 1.400 types de fromages différents dans le monde, avec une grande variété de textures, de saveurs et d'arômes. Ces caractéristiques sont attribuées au développement de communautés microbiennes complexes et spécifiques selon la technologie employée et les ingrédients utilisés, mais aussi selon les facteurs locaux tels que l'origine du lait et les pratiques agricoles [5-7].

1.1. Classification des fromages

La diversité et la complexité des variétés de fromage génèrent des difficultés en ce qui concerne sa classification et caractérisation. L'approche « européenne » (plutôt française) utilise les procédés technologiques comme critères de classification [8] et le modèle « anglo-saxon » est principalement basé sur les propriétés de la texture (fermeté) [9]. Bien que la catégorisation proposée par Lenoir *et al.* (1985) soit largement utilisée [8], elle est particulièrement adaptée aux fromages français et ne représente pas une vision globale de la production fromagère. Par exemple, il y a deux points principaux dont cette version fait abstraction: (i) la coagulation par la chaleur et l'acidification (ii) matières premières telles que le lactosérum, la crème et le colostrum pour la production de fromages. Almena-Aliste et Mietton (2014) ont donc proposé des modèles plus intégratifs de classification afin de mieux décrire la diversité des fromages, en tenant compte des caractéristiques technologiques, microbiologiques, chimiques et sensorielles [10]. Ici, nous associons le modèle de Lenoir *et al.* [8] et le diagramme proposé par Almena-Aliste et Mietton [10], afin de fournir un panorama des catégories de fromage existantes ([Fig. 1](#)).

La coagulation du lait, première étape de la fabrication du fromage, correspond à une déstabilisation de l'état micellaire originel des caséines du lait et peut être réalisée principalement de deux manières (i) par voie fermentaire, à l'aide de bactéries lactiques (ii) par voie enzymatique, à l'aide d'enzymes coagulantes, en particulier la présure [11]. Dans les techniques fromagères, les deux méthodes sont normalement utilisées ensemble, sous forme d'une coagulation mixte.

En général, les fromages avec coagulation lactique et/ou enzymatique représentent ~75% de la production totale de fromage et presque 100% des fromages affinés [12]. Les fromages avec large dominance de bactéries lactiques se caractérisent par une coagulation lente (environ 16 heures) et une synérèse très courte (le caillé est coupé peu avant le moulage). Après un égouttage et un salage à sec, les fromages à pâtes lactiques peuvent être affinés, soit par l'ajout de moisissures (comme *Penicillium candidum* au fromage Chaource), soit par des lavages d'eaux salées ou non-salées (fromages à croûte lavée).

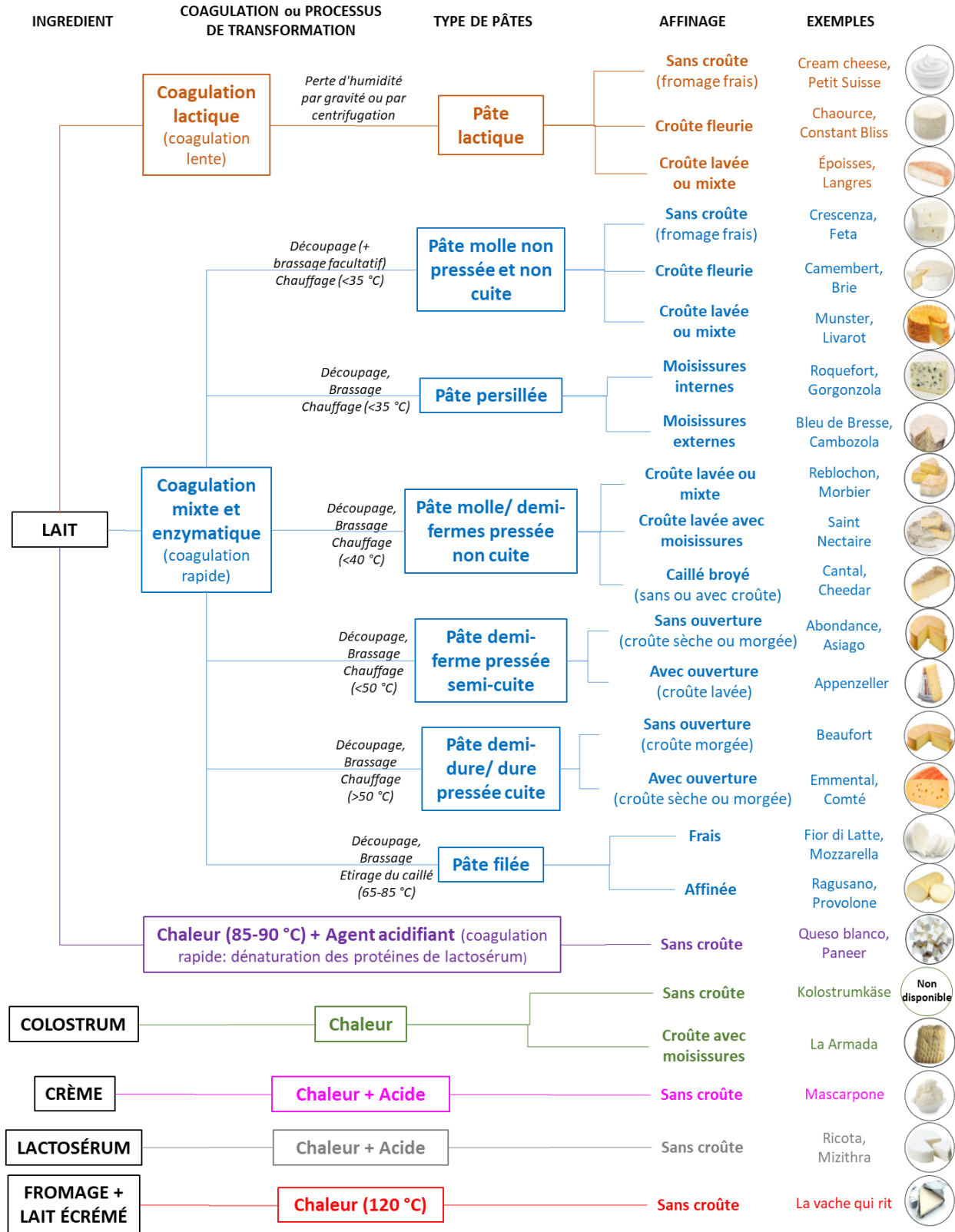


Figure 1. Classification des fromages basée sur la technologie et les caractéristiques microbiologiques (Adpté de Almena-Aliste & Mietton [10] et Lenoir *et al.* [8]).

D'un autre côté, il existe un grand groupe de fromages qui sont fabriqués avec la coagulation enzymatique et mixte. C'est le cas des pâtes non-cuites, semi-cuites et cuites, ainsi que des pâtes persillées et filées. Les fromages à pâtes non-cuites sont chauffés à températures maximales de 40°C et peuvent être pressés ou non-pressés. Les non-pressés ont une pâte molle et peuvent être classés sans croûte (Feta), croûte fleurie (Brie) ou croûte lavée (Munster/Livarot). Les fromages à pâtes pressés peuvent avoir une croûte lavée sans ou avec moisissures (Morbier et Saint Nectaire, respectivement). D'autre part, les pâtes semi-cuites (<50°C) et cuites (>50°C) peuvent être classés en pâtes avec ou sans ouvertures et leurs croûtes peuvent être naturellement séchées, lavées ou frottées avec une solution morge (à base de saumure), comme dans le cas du Comté et du Beaufort.

Les fromages à pâte persillée sont fabriqués à partir de lait de vache ou de brebis et les plus connus sont, respectivement, le Gorgonzola et le Roquefort. Ils ont un aspect « persillé » car, après la phase d'acidification lactique et l'ajout de présure, le fromage estensemencé de moisissures bleues telles que *Penicillium glaucum* ou *P. roqueforti*. Dans ce groupe de fromages bleus, le Bleu de Bresse, par exemple, en plus d'avoir une pâte persillée, peut être aspergé de *Penicillium camemberti*, après le moulage, résultant en une croûte fleurie. D'un autre côté, les variétés de fromage à pâte filée sont produites à partir de lait coagulé avec de la présure et acidifié à l'aide de *Streptococcus thermophilus* et d'un *Lactobacillus* spp. thermophile comme ferments [12]. Lorsque le pH du caillé atteint 5,1-5,3, celui-ci est chauffé, malaxé et étiré dans de l'eau chaude ou de la saumure diluée (65-85°C). Les fromages à pâte filée peuvent être consommés frais (comme la Mozzarella) ou affinés, ou encore fumés (comme le Provolone).

En revanche, les fromages coagulés à l'acide et/ou à la chaleur représentent ~25% de la production de fromage et sont généralement consommés frais [12]. Ils peuvent être fabriqués à partir de diverses matières premières telles que le lait, le lactosérum, le colostrum ou la crème. Le fromage Ricotta, par exemple, est produit à partir de lactosérum par coagulation induite par la chaleur (85-90°C) et de certains agents acidifiants (comme le jus de citron ou le vinaigre). D'autre part, le fromage Mascarpone est produit à partir de crème comme ingrédient, selon un procédé similaire à celui de la Ricotta, mais en utilisant des températures de cuisson légèrement plus élevées et une teneur en sel plus faible. Les fromages fondus, tels que « La vache qui rit », sont fabriqués en mélangeant des fromages naturels broyés (comme l'Emmental, le Comté, le Gouda,

etc) avec des sels émulsifiants et d'autres ingrédients laitiers, et en chauffant le mélange sous vide (120°C) sous agitation constante, jusqu'à obtention d'un mélange homogène.

Tous ces différents types de fromage sont composés de différents microorganismes d'origines diverses (ingrédients, fermentsensemencés, saumures, équipements et matériels de la fromagerie, caves d'affinage, etc). Ces microorganismes peuvent contribuer à la vaste diversité des caractéristiques organoleptiques des cœurs et des croûtes/surfaces des fromages.

1.2. Diversité microbienne des fromages

Au cours des dernières années, de nouveaux outils ont permis d'étudier les fromages d'une manière auparavant inconcevables. Ainsi, l'utilisation de ces nouvelles technologies, comme le séquençage à haut débit, a révolutionné le domaine de l'écologie microbienne, en permettant une identification plus exhaustive des microorganismes, y compris ceux qui sont difficiles à cultiver et/ou qui sont présents en faible abondance [13]. Cette science émergente a apporté de nouvelles perspectives sur la biodiversité microbienne, en particulier, pour les fromages artisanaux [3, 4, 14] et, par conséquent, en développant une connaissance et un intérêt accrus pour les aliments traditionnels plus anciens.

Ces microorganismes présents dans les fromages peuvent être ajoutés par l'homme (ferments), selon la technologie ou les caractéristiques souhaitées, ou ils peuvent provenir de l'extérieur tout au long de la chaîne de production (microorganismes adventices) (Fig. 2). Les deux procédés, intentionnels ou non, en combinaison avec les transformations chimiques et biochimiques, définissent les caractéristiques et l'identité du fromage [15].

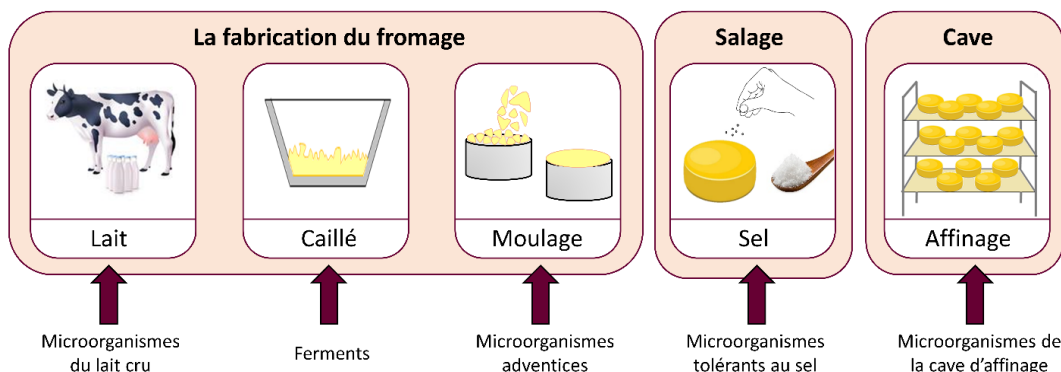


Figure 2. Possibles sources de développement de communautés microbiennes spécifiques dans le processus de production et d'affinage du fromage (Adapté de Wolfe *et al.*[3]).

1.2.1. Les microorganismes ajoutés intentionnellement

Les ferments (encore appelés agents de fermentation ou levains) sont des souches bien définies et caractérisées, ajoutés intentionnellement, utilisés lors de la fabrication du fromage pour contrôler la fermentation et assurer la régularité de la production du produit [1]. La norme ISO 27205:2010 [16] définit une culture initiale pour la production de fromage comme suit: « culture préparée qui contient une ou plusieurs souches de microorganismes en nombre élevé (en général plus de 10^8 UFC/g ou ml de bactéries viables) qui sont ajoutées pour provoquer une réaction enzymatique souhaitable (par exemple, fermentation du lactose entraînant la production d'acide, dégradation de l'acide lactique en acide propionique, ou autres activités métaboliques directement liées aux propriétés spécifiques du produit) ».

Les cultures microbiennes d'origine alimentaire se sont directement ou indirectement insérées dans plusieurs cadres réglementaires au cours des dernières années, que ce soit en mettant en évidence l'historique d'utilisation, les aliments traditionnels ou la présentation d'une parfaite innocuité pour le consommateur et l'environnement (GRAS: *Generally recognized as safe*) [17]. En 2002, pour classer les ferments traditionnellement utilisés comme ingrédients alimentaires sécurisés, l'*International Dairy Federation* (IDF) - en collaboration avec l'*European Food and Feed Cultures Association* (EFFCA) - a compilé un inventaire des microorganismes tenant en compte la littérature scientifique documentée de leur utilisation dans l'alimentation (Bulletin de l'IDF n° 377/2002). Cet inventaire est devenu une référence pour l'utilisation pratique des souches alimentaires. En 2012, Bourdichon *et al.* [17] ont mis à jour cet inventaire des microorganismes en se concentrant sur les aliments fermentés, y compris le fromage. Les principaux microorganismes qui peuvent être utilisés lors de la production de fromage comme ferments sont brièvement présentés ci-dessous.

Bactéries lactiques

Les bactéries de l'acide lactique (LAB) sont parmi les microorganismes les plus étudiés dans le monde. Actuellement, avec les développements des technologies de séquençage de la nouvelle génération, des milliers de génomes sont disponibles et, en conséquence, de nombreuses caractéristiques importantes des souches LAB sont connues, y compris les aspects de la fermentation du sucre, de la formation d'arômes, de la production de substances texturées, des réponses au stress, de la colonisation et de la survie chez l'hôte, des interactions entre cellules et

de la pathogénicité [13]. Une recherche du terme « *lactic acid bacteria* » dans le titre, les mots clés et le résumé dans la base de données scientifique [Scopus](#) a donné environ 24.895 résultats d'études (novembre 2020), montrant que les LAB ont reçu beaucoup d'attention de la part de la communauté scientifique. De plus, en utilisant « *lactic acid bacteria* » ET *food*, « *lactic acid bacteria* » ET *cheese*, et « *lactic acid bacteria* » ET *environment* comme termes de recherche, 10.802, 2.359 et 1.293 documents ont été retrouvés, indiquant que les aliments sont largement étudiés en association avec les LAB.

Ainsi, au cours des trois dernières décennies, les recherches sur les espèces LAB ont contribué à une sélection et application bien conçues de ferments pour l'industrie alimentaire [18, 19]. En ce qui concerne les fromages, la plupart ne peut pas être fabriquées sans ajout de certaines bactéries lactiques dont les principales fonctions sont d'acidifier le produit pendant la fabrication et de générer des modifications biochimiques pendant l'affinage. L'ajout intentionnel de ces bactéries peut aussi contribuer à développer le goût ou la qualité des textures, ajoutant de la valeur aux produits alimentaires [20].

Les LAB - telles que *Lactococcus lactis* et *Streptococcus thermophilus* - sont indispensables à la fabrication de la grande majorité des fromages. Elles possèdent généralement un métabolisme homo-fermentaire et génèrent l'acide lactique comme principal produit final [21, 22]. Les « *non-starter* » LAB (NSLAB) constituent également une partie importante de la flore lactique secondaire qui se développe au cours de l'affinage des fromages et sont utilisées comme ferments de fromagerie depuis les années 90 [23]. Ces bactéries correspondent principalement à des lactobacilles hétéro-fermentaires facultatifs, comme les espèces suivantes: *Lactocaseibacillus paracasei* et *casei*, *Lactiplantibacillus plantarum* et *Pediococcus acidilactici* [5]. Les métabolismes hétéro-fermentaires génèrent certains métabolites, autres que l'acide lactique, tels que l'éthanol, le dioxyde de carbone ou l'acide acétique, et contribuent à améliorer les arômes du fromage [5, 22, 24]. En général, les fournisseurs vendent des mélanges de ferments homo et hétéro-fermentaires avec des proportions différentes suivant les caractéristiques à privilégier.

Les ferments ajoutés pendant la fabrication du fromage peuvent également être classés en fonction de leur température de croissance optimale (mésophiles ou thermophiles). Par définition, le terme « mésophile » désigne les organismes dont la température de croissance optimale se situe entre 20 et 37°C [25]. Les cultures mésophiles sont normalement utilisées dans la fabrication de fromages frais, de pâtes molles et de pâtes semi-cuites [26]. D'autre part, pour les fromages à pâtes

cuites, l'emploi des cultures thermophiles est indiqué. Elles sont composées de bactéries qui se développent à températures plus élevées (entre 40 et 50°C) [27]. Ces cultures ont la particularité de donner de la souplesse aux pâtes pressées ou générer des enzymes texturants en pâtes filées [28, 29]. Comme exemple, *Lactococcus lactis* ssp. *lactis* et *L. lactis* ssp. *cremoris* sont les principaux ferments mésophiles largement utilisés dans la fabrication du fromage. Ils sont fréquemment combinés avec d'autres cultures mésophiles (*Leuconostoc mesenteroides*, *L. pseudomesenteroides*) ou thermophiles (*Streptococcus thermophilus*) [5, 27].

Cultures d'affinage

Au-delà des bactéries lactiques (LAB ou NSLAB), d'autres cultures fromagères peuvent contribuer à la définition des caractéristiques des fromages. Dans certains produits traditionnels à croûte lavée, par exemple, les fromages plus anciens sont frottés au contact de fromages jeunes pour transférer leurs microorganismes [30]. Aujourd'hui, cependant, les mélanges commerciaux de bactéries et de levures sont plus couramment utilisés dans les productions à large échelle afin de produire un produit plus standardisé. Les kits de microorganismes d'affinage peuvent être constitués de champignon ascomycètes telles que *Penicilium* spp. et *Geotrichum candidum*, de la levure *Debaromyces hansenii* et des bactéries comme *Brevibacterium linens/aurantiacum*, *B. casei*, *Staphylococcus xylosum*, *S. equorum* et *Glutamicibacter arilaitensis* [31, 32]. La composition et la proportion de microorganismes dans chaque mix dépend du type de fromage fabriqué et des caractéristiques souhaitées. Par exemple, le *Penicilium roqueforti* est utilisé dans la fabrication de fromages bleus et un mélange de *G. candidum*, *D. hansenii*, *B. linens*, *G. arilaitensis* et *S. xylosum* est commercialisé pour la fabrication de fromages à croûte lavées [32]. Ces dernières cultures sont souvent utilisées dans les fromages jeunes et peuvent être pulvérisées sur la surface du fromage ou incorporées à la saumure [33].

Toutes les espèces bactériennes mentionnées ci-dessus appartiennent au groupe des Gram-positifs et peuvent être utilisées comme cultures d'affinage, bien qu'elles puissent également être fortuites. À ce jour, la seule bactérie Gram-négatif utilisée comme culture commerciale est l'*Hafnia alvei* [5], notamment pour les fromages type Camembert au lait cru [34, 35]. La présence de certaines souches de cette espèce augmente le niveau de composés soufrés volatils et peut, conséquemment, contribuer au goût du fromage [36].

Néanmoins, selon Delbès *et al.* (2015), les microorganismes ajoutés intentionnellement dans les fromages peuvent entrer en compétition avec leur microbiote endogène et se montrer faiblement adaptés aux étapes de fabrication de ce produit [6]. Rea et ses collègues (2007) ont évalué la production de six lots d'un fromage à croûte lavée irlandais (Gubbeen) et ont noté que des souches commerciales, délibérément inoculées, peuvent être présentes en tant que sous-populations, mais ne font pas partie de la microflore dominante [37]. En effet, des études menées au cours des 20 dernières années ont montré que les levures et/ou les ferments bactériens ajoutés intentionnellement, notamment dans les fromages à croûte lavée, ne se développent pas nécessairement lors de la fabrication du fromage et qu'une flore adventice peut être dominante à la surface de ces produits fermentés [31, 38-40].

1.2.2. Les microorganismes adventices

Le lait, principal ingrédient de la production de fromage, est riche en nutriments, présente une humidité élevée et un pH presque neutre, ce qui en fait un excellent substrat de croissance non seulement pour les ferments, mais aussi pour les microorganismes adventices [41]. Bien que le lait sorte de la mamelle des animaux, essentiellement stérile, il peut être contaminé par des microorganismes provenant du trayon et du sphincter, de l'environnement de la ferme, des équipements de traite et des récipients de conditionnement [42]. Dans les temps anciens, avant l'utilisation de la réfrigération, les bactéries mésophiles Gram-positives, en particulier les LAB comme *Streptococcus*, *Enterococcus*, *Lactobacillus*, *Leuconostoc*, *Lactococcus* et *Pediococcus*, dominaient tout développement bactérien dans le lait et contribuaient donc à leur acidification [43]. Avec l'utilisation des récipients à refroidissement rapide et au stockage réfrigéré, les basses températures ($<7^{\circ}\text{C}$) ont réduit les populations de bactéries lactiques dans le lait et ont favorisé la croissance de bactéries Gram-négatives psychrophiles telles que *Pseudomonas* et *Acinetobacter* [44, 45]. La forte activité protéolytique et lipolytique de certaines espèces de ces genres peut entraîner de nombreux inconvénients pour le fromage, notamment des odeurs désagréables, de l'amertume et une saveur rance. En outre, des coliformes tels que *Citrobacter*, *Escherichia*, *Enterobacter* et *Klebsiella* font également partie de la flore psychrophile et peuvent provoquer des saveurs désagréables ainsi que des défauts dans le lait et les fromages [45, 46].

D'autre part, le microbiote indigène présent dans les matières premières ou dans l'environnement de production, comme les caves d'affinage, peut produire des fromages

d'excellente qualité. Mounier *et al.* (2005) confirment l'importance de la flore adventice et de leur présence dans les fromages affinés [40]. Les chercheurs ont isolé des microorganismes à partir de quatre types de fromages irlandais à croûte lavée (Gubeen, Durrus, Ardrahan et Mileens), dont aucun n'appartenait aux cultures commerciales d'affinage, ajoutées intentionnellement à la surface des fromages. Ils ont obtenu *Debaryomyces hansenii*, comme levure dominante, dans les quatre fromages évalués, et une grande diversité bactérienne, parmi laquelle des espèces appartenant aux genres *Agrococcus*, *Arthrobacter*, *Bacillus*, *Brevibacterium*, *Corynebacterium*, *Halomonas*, *Microbacterium*, *Micrococcus*, *Staphylococcus* et *Vibrio*.

Grâce à des techniques indépendantes de la culture, comme la métagénomique, une vue plus complète de la biodiversité peut être mieux établie dans les écosystèmes fromagers. En 2014, par exemple, une étude américaine menée dans le laboratoire de Rachel Dutton, a analysé par metabarcoding 137 croûtes de fromages provenant de dix pays différents et ils ont constaté qu'au moins 60% des genres bactériens et 25% des champignons présents dans ces produits sont des microorganismes adventices [3]. Parmi les groupes non inoculés, ils ont identifié deux genres bactériens, *Nocardiopsis* et *Yaniella*, qui n'avaient jamais été signalés dans les écosystèmes alimentaires microbiens. En outre, ils ont montré que les protéobactéries telles que les *Halomonas*, *Pseudoalteromonas*, *Psychrobacter* et *Vibrio* sont très répandues dans les communautés fromagères, ce qui a également été observé dans d'autres études métagénomiques sur les croûtes de fromage [3, 4, 47]. De plus, Dugat-Bony *et al.* (2016) ont évalué 12 variétés de fromages français avec la métagénétique (amplicon bactérien et fongique), et ont également souligné l'importance des microorganismes indigènes dans l'écosystème fromager [48]. Alors que la plupart des échantillons étaient dominés par les espèces fongiques *Geotrichum candidum*, *Debaryomyces hansenii* et *Candida sake*, la composition de la communauté bactérienne globale était beaucoup plus variable parmi les échantillons. Les éventuelles bactéries adventices trouvées dans le cœur et croûtes des fromages étudiés appartenaient aux genres *Halomonas*, *Pseudoalteromonas*, *Pseudomonas*, *Psychrobacter* et *Psychroflexus*. La prédominance de ces bactéries psychrophiles dans les fromages peut être due aux conditions de stockage appliquées pour le transport et la distribution aux supermarchés (température de 4-8°C pendant des jours ou des semaines) [48].

Ainsi, ces études confirment que la microflore adventice qui se développe dans les fromages peut être assez complexe, selon l'endroit où ils sont affinés et la façon dont ils sont préparés. Les sources potentielles de ce microbiote résidant dans les fromages sont liées à l'environnement de la

ferme ou de la cave d'affinage, aux récipients et équipements utilisés, ainsi qu'aux ingrédients utilisés tels que le lait et le sel ajoutés pendant la production [5, 49-51].

2. Le sel dans l'alimentation

Encadré 3 : Le sel comme agent de sélection des microorganismes

Le sel joue généralement un rôle central dans la production d'aliments fermentés, qui non seulement ont une longue durée de conservation, mais développent également des caractéristiques technologiques comme la saveur et la texture, faisant ainsi partie du patrimoine culturel de plusieurs pays. La teneur en sel dans les fromages peut varier de 0,3 à 7% et cet environnement « salé » peut favoriser la croissance de microorganismes tolérants au sel.

En tant que produit naturel, le sel (chlorure de sodium, NaCl) a été récolté depuis l'Antiquité sur des lacs et des oasis, ainsi qu'au long des littoraux, puis de manière plus industrielle dès la protohistoire du 5-6^{ème} millénaire avant J.-C., dans des mines telles que Hallstatt (Autriche), Lunca (Roumanie), Duzdagi (Azirbadjan) ou Shabwa (Yémen) [52-54]. Aujourd'hui, il existe trois grandes méthodes pour obtenir du sel : (i) l'évaporation de l'eau du mer ; (ii) l'extraction des cristaux de sel gemme et (iii) la création de saumures de sel (sel ignigène) [55, 56].

Le sel représente environ 3,5 % des océans du monde et peut être produit naturellement lorsque les étangs et les baies peu profondes s'assèchent au soleil [57]. Lors de la production de sel marin à l'échelle industrielle, l'eau du mer est placée dans de grands bassins pour permettre une évaporation efficace, face au soleil et au vent [58, 59]. Ainsi, pour obtenir une évaporation complète de l'eau, le sel marin est, de préférence, fabriqué sous climats secs et dans les pays à faible pluviométrie, comme le Brésil, le Cap-Vert, la Colombie, le Mexique, l'Afrique du Sud, le Venezuela et l'Australie occidentale [59]. Le sel de mer peut également être produit selon des techniques anciennes et artisanales, à une échelle beaucoup plus petite, comme la Fleur de sel. Ce sel léger et gastronomiquement réputé, est fabriqué dans de petits lagons en France, seulement, pendant les mois d'été, de juin à août [60, 61].

Le sel gemme (aussi appelé halite) s'est formé lors de l'évaporation des mers géologiques il y a plusieurs millions d'années, et est présent dans les couches rocheuses inférieures de la surface de la Terre [62]. Il est extrait à la dynamite, de la même manière que tout autre minéral, ou d'une manière plus artisanale à la main, et est ensuite broyé en différentes tailles selon les besoins respectifs. Le sel gemme alimentaire est également connu sous le nom de sel de l'Himalaya, de sel rose ou de sel de Khewra (importante mine de sel, au Pakistan) et c'est l'un des types de sel les moins transformés et disponibles sur le marché [63].

Le sel ignigène est obtenu de gisements souterrains et est dissous par injection d'eau douce afin d'obtenir une saumure remontée en surface pour être évaporée [64]. Alors que l'océan est une saumure naturelle, l'extraction hydraulique (ou extraction par dissolution) du sel consiste à pomper de l'eau souterraine pour dissoudre les dépôts de sel et créer une saumure. Cette saumure est ensuite évaporée pour créer le sel. La saumure salée peut être traitée avant l'évaporation pour réduire sa teneur en minéraux, ce qui donne un cristal de chlorure de sodium presque pur. Comme cette méthode est peu coûteuse, a un rendement élevé et produit un sel très propre, la plupart du sel de table est produit de cette façon [65].

Selon l'U.S. Geological Survey (2020), la production mondiale de sel est dominée spécialement par trois pays : la Chine (20%), les États-Unis (14%) et l'Inde (10%) [66]. Sur les 290 millions de tonnes de sel produites annuellement, environ 43% sont utilisés pour le déglacage des routes, 37% pour l'industrie chimique et seulement 9% sont utilisés pour la consommation humaine [66]. Alors que les sels utilisés à des fins industrielles sont principalement obtenus à partir de l'exploitation minière, la plupart des sels de table sont produits à partir de la saumure, tandis que les sels spéciaux ou gastronomiques sont presque toujours produits par évaporation de l'eau de la mer. Indépendamment de la manière dont il est extrait et de son origine, le sel est un ingrédient largement utilisé dans l'alimentation en raison de son rôle technologique, de préservation et d'appréciation sensorielle.

2.1. Le sel comme conservateur

Le processus de salage est utilisé comme principale méthode de conservation depuis l'antiquité [67], et il peut se faire de deux manières : par l'ajout de cristaux de sel ou par la saumure. Les Sumériens, par exemple, utilisaient le sel pour conserver toutes sortes d'aliments, y compris le poisson, les graisses, la viande, l'orge et le blé, en 3.000 avant J.-C. [68].

Le sel est efficace comme conservateur car il réduit l'activité de l'eau (a_w) des aliments, qui est un critère de la disponibilité d'eau « libre » d'une matrice alimentaire, susceptible d'intervenir dans des réactions chimiques, biochimiques ou microbiologiques [69]. Alors que les aliments ayant un a_w élevé ($>0,95$) comprennent la viande fraîche, les œufs et les légumes frais, des valeurs d' a_w faibles ($<0,90$) peuvent être trouvées dans les fromages du type Parmesan, les jambons et les pains [70]. Ainsi, le NaCl peut réduire l' a_w et conséquemment les taux de multiplication microbienne dans les aliments, grâce à la capacité des ions sodium et chlorure à s'associer aux molécules d'eau et à provoquer un choc osmotique dans les cellules microbiennes, entraînant une perte d'eau et donc la mort des cellules ou le ralentissement de leur développement [71-73]. De plus, des études suggèrent que, pour certains microorganismes, le sel peut limiter la solubilité de l'oxygène, interférer avec les enzymes cellulaires, ou forcer les cellules à dépenser de l'énergie pour exclure les ions sodium de la cellule, ce qui peut réduire leur croissance [74].

Le sel reste un ingrédient couramment utilisé pour assurer un environnement résistant à la détérioration et pour empêcher la croissance des agents pathogènes, ce qui renforce la sécurité et la *shelf-life* des produits, tels que le jambon, le poisson et le fromage. Cependant, au-delà du simple ajout de sel, d'autres méthodes de conservation sont normalement combinées afin de permettre l'obtention d'un produit de qualité, stable et sécurisé [69]. Par exemple, l'utilisation du sel dans la formulation de la viande prête à consommer, en combinaison avec un conservateur chimique (comme le nitrate), des valeurs de pH et de température adéquates, peut réduire le risque de germination des spores d'espèces qui provoquent des intoxications alimentaires comme le *Clostridium perfringens* [75, 76] et le *Clostridium botulinum* [77, 78].

Ainsi, alors que des niveaux de sel élevés inhibent complètement la croissance de la plupart des microorganismes, des niveaux de sel modérés engendrent un environnement inhospitalier pour la plupart des agents pathogènes et peuvent aussi favoriser les aspects organoleptiques et technologiques bénéfiques pour divers aliments fermentés.

2.2. Le rôle technologique du sel dans la production des aliments

Bien que de nombreuses industries alimentaires cherchent notamment à réduire la teneur en sel de leurs produits, les aspects technologiques et sensoriels, tels que la texture et le goût, peuvent affecter les formulations [79, 80] et influencer l'acceptation du consommateur [81]. En ce

qui concerne l'aspect organoleptique, le sel peut faciliter la libération de certaines molécules, conférant des arômes perceptifs au niveau du palais et diminuer la diffusion du goût amer [82, 83].

Dans les produits transformés à base de viande, par exemple, le sel peut être un élément essentiel qui contribue à la texture. Ruusunen *et al.* (2001) ont rapporté que le jambon cuit dont la teneur en sel ajoutée était inférieure à 1,4% présentait des pertes à la cuisson plus importantes que les jambons dont la teneur en sel était supérieure à 1,7% [84]. Des auteurs soulignent que dans les produits à faible teneur en sel et à forte teneur en eau, il est nécessaire d'ajouter plus de protéines ou d'autres ingrédients fonctionnels pour augmenter le rendement [80, 84].

Le NaCl aussi facilite la manipulation et la transformation de nombreux produits fermentés, comme les pains, les légumes et les fromages [70]. Dans le pain, le sel a comme fonction de rendre le gluten plus stable et moins extensible et collant, en plus de réguler le taux de fermentation. Sans sel, la fermentation de la levure est très rapide, produisant de grandes cellules d'air irrégulières dans la structure de la mie. Une fermentation rapide peut également consommer énormément de sucre, ingrédient essentiel pour les réactions de brunissement telles que la caramélisation et la réaction de Maillard qui contribuent à la couleur du pain [85]. Dans les légumes fermentés tels que la choucroute et les *pickles*, le sel favorise la texture croquante car il diminue l'activité de la pectinase, une enzyme qui rend les végétaux pâteux. En outre, le sel peut ralentir le processus de fermentation, ce qui permet un développement complet du goût et la réduction du risque lié à la présence de microorganismes indésirables [69]. Le fromage est un aliment dont le sel peut aussi réguler la fermentation et permettre la croissance de microorganismes tolérants au sel. En plus, le NaCl peut affecter directement le corps/la texture du fromage en modifiant la structure des protéines ou, indirectement, en éliminant l'humidité. En fait, la réduction de la teneur en NaCl dans les fromages à pâtes semi-rigides, tels que le Cheddar, entraîne une augmentation de l'amertume et le côté désagréable du goût, ainsi qu'une réduction de la rigidité [86-88].

2.3. Le sel et la salage des fromages

En général, les différents types de fromage présentent une très large gamme de sel. Le [Tableau 1](#) a été adaptée à partir des données provenant de l'Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail (Anses, 2020) [89] et présente la teneur en sel de certains fromages vendus en France, sachant que les valeurs peuvent varier selon chaque fromager.

Tableau 1. Teneur en sel, par ordre croissant, de certains fromages en fonction de leurs respectives catégories (Adapté de Anses [89]).

Catégorie	Fromage	NaCl (g/100 g)
Blancs	Faisselle	0,11
Blancs	Fromage blanc nature	0,11
Spécialités	Ricotta	0,30
Spécialités	Mozzarella	0,49
Pâte pressée	Emmental	0,75
Pâte pressée	Comté	0,80
Pâte pressée	Gruyère	0,80
Pâte molle	Langres	0,99
Pâte molle	Crottin de chèvre	1,01
Pâte molle	Reblochon	1,27
Pâte pressée	Beaufort	1,27
Pâte molle	Pont l'Évêque	1,30
Pâte pressée	Morbier	1,41
Pâte molle	Pélarion	1,43
Pâte persillée	Bleu de Gex	1,44
Pâte molle	Camembert	1,45
Pâte molle	Livarot	1,50
Pâte molle	Brie	1,51
Pâte pressée	Grana Padano	1,56
Pâte pressée	Parmesan	1,57
Pâte pressée	Cheddar	1,61
Pâte pressée	Ossau-Iraty	1,68
Pâte pressée	Saint-Nectaire	1,70
Pâte molle	Époisses	1,76
Pâte persillée	Gorgonzola	1,77
Pâte molle	Munster	1,78
Pâte pressée	Cantal entre-deux	1,85
Pâte pressée	Gouda	2,00
Pâte pressée	Provolone	2,19
Pâte pressée	Edam	2,22
Spécialités	Feta	2,27
Pâte pressée	Mimolette	2,84
Pâte persillée	Bleu d'Auvergne	2,85
Pâte persillée	Roquefort	3,22
Pâte pressée	Pecorino	4,73

En dehors des fromages blancs (sans ajout de sel), la Ricotta et la Mozzarella sont les fromages moins salés avec un taux de 0,3 et 0,5%, respectivement. La majorité des fromages varient entre 1-2% de sel et certains, comme le Roquefort et le Pecorino, contiennent plus de 3% de sel dans leur composition. Il existe aussi des fromages, comme le Domiati et le Sikma, très consommés en Égypte et en Turquie, respectivement, qui peuvent contenir jusqu'à 7 % (p/p) de sel [90, 91].

Pour incorporer le sel au fromage, lors de la production, quatre approches différentes peuvent être utilisées. Une première méthode d'application consiste à mélanger le sel directement avec les particules de caillé, avant qu'il ne soit pressé, pour former le fromage final [92] ([Fig. 3](#)). L'ajout de sel aux caillés de fromage génère une force motrice osmotique qui attire le lactosérum vers la surface, où il est libéré [92]. Ainsi, au-delà des étapes de fabrication du fromage comme la découpe, l'égouttage et le pressage, le salage est une autre étape utilisée pour réduire l'humidité du fromage. Cette technique permet d'incorporer uniformément de fortes concentrations de sel dans toute la masse et d'éliminer efficacement le lactosérum du caillé au cours du processus. L'expulsion du lactosérum obtenue par cette méthode de salage permet également aux fromagers de produire des variétés à faible teneur en humidité, comme le Cheddar, sans avoir besoin de températures de cuisson élevées, ce qui constitue un grand avantage pratique [11].

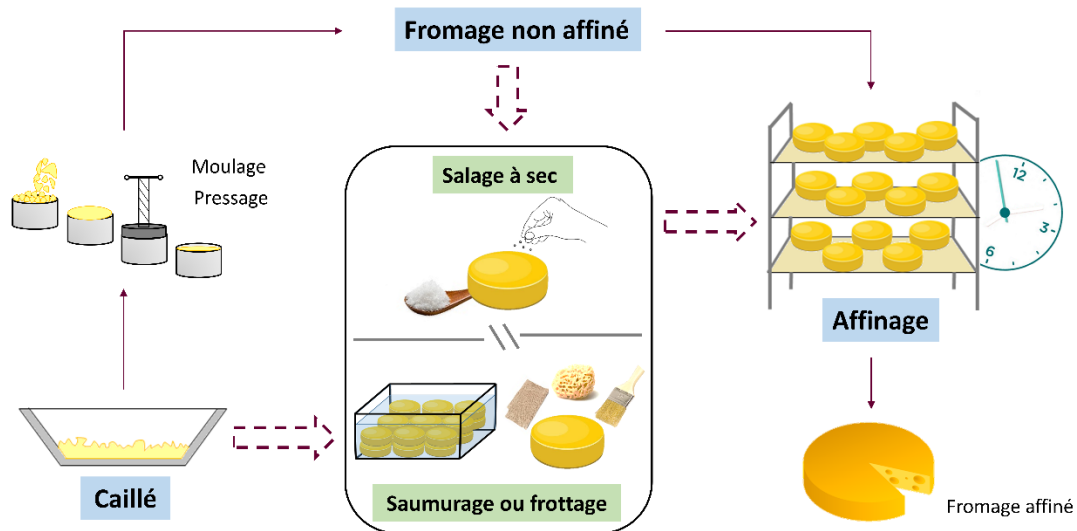


Figure 3. Étapes de la fabrication du fromage impliquant l'ajout de sel (bleu) et les méthodes de salage (vert).

La deuxième méthode peut être appliquée au fromage avant ou pendant l'affinage, soit par ajout de sel en cristaux ou par saumure, et ces procédés peuvent être utilisés une ou plusieurs fois et à des différents échelles de temps ([Fig. 3](#)). Le sel sec peut être frotté sur la surface des fromages, où il se dissout à l'intérieur et, parallèlement, l'humidité est transférée à la surface et s'évapore. Les fromagers traditionnels appliquent le sel de manière répétée avec un frottement légèrement abrasif afin de développer une croûte lisse, dense et imperméable qui rend le fromage très stable et durable. Bien que cette méthode fonctionne bien pour les petits fromages (comme le Limburger), la

technique devient problématique pour les gros fromages, car le salage à sec déshydrate trop rapidement la surface [93]. Par conséquent, la couche extérieure salée peut devenir épaisse et empêcher la migration du sel vers l'intérieur du fromage ([Fig. 4](#))



Figure 4. Fromage à croûte lavée (Adapté de Wolfe *et al.* [3]).

La troisième méthode consiste à submerger le fromage dans une saumure concentrée (qui peut varier de 10 à 20% de sel) [11]. Ce salage est particulièrement utile pour la fabrication de grands fromages à croûte lavée, car il permet une plus grande absorption du sel tout en déshydratant progressivement la surface en vue de la formation de la croûte. L'immersion en saumure entraîne des gradients importants et persistants de sel et d'humidité, s'étendant de la surface vers le centre du fromage, ce qui affecte l'écologie microbienne de manière localisée: les microorganismes sensibles au sel peuvent se trouver en abondance dans le centre (faible teneur en sel) et les types tolérants en surface (forte teneur en sel) [11]. Cette méthode est utilisée pour les fromages comme le Livarot et l'Ossau Iraty.

Parallèlement à l'immersion dans la saumure, la quatrième méthode de salage consiste à frotter le fromage pendant l'affinage avec une solution appelée morge, dont les ingrédients de base sont l'eau et le sel. La quantité de sel à incorporer dans l'eau est très variable (5-35%) et dépend du type de fromage et de la charge de contamination dans les caves d'affinage. Il est également possible de diminuer le pH de la morge (aux alentours de 5,0-5,2) en ajoutant un acide (comme du vinaigre blanc) afin d'éliminer les levures indésirables et de stimuler le développement de levures tolérantes aux acides [94]. Le frottement de la solution de morgeage dans les fromages peut se faire avec un torchon, un pinceau/brosse ou une éponge ([Fig. 3](#)), en commençant par les fromages les plus anciens et en terminant par les plus jeunes, sans croûte. C'est ainsi que les microorganismes s'incorporent et donnent l'originalité de la solution, qui peut être conservée plusieurs jours et contribuer à des frottages successifs. Cette méthode est très artisanale et fréquente dans les petits ateliers, mais elle tend à disparaître dans les grandes industries, qui ont tendance à privilégier une

nouvelle solution de frottage à chaque traitement. Dans ce cas, l'ajout de souches commerciales à la morge initiale peut être utile pour permettre l'ensemencement de la surface des fromages et ainsi favoriser la formation de la croûte. Ces cultures peuvent être proposées sous forme lyophilisée ou liquide par les fournisseurs de ferments et peuvent se constituer principalement des levures *Debaryomyces hansenii* et *Geotrichum candidum*, ainsi que de bactéries aérobies non lactiques telles que les corynebactéries (en particulier *Brevibacterium linens/aurantiacum*) et les microcoques [94].

La phase de salage, lors de la fabrication du fromage, pourra donc générer une forte concentration de sel, favoriser la croissance de microorganismes adaptés à ces environnements et favoriser le développement des propriétés organoleptiques comme le goût, l'apparence et la texture du produit final. Une meilleure compréhension de l'origine et de la manière dont ces organismes évoluent, pendant les différentes étapes du développement du fromage, pourrait être d'un grand intérêt pour déterminer comment le sel pourrait favoriser la composition du microbiote du fromage.

2.3.1 Quels types de sel pour la fromagerie?

Selon le Décret n° 2007-588 du 24 avril 2007, relatif aux sels destinés à l'alimentation humaine, le sel de qualité alimentaire est « un produit cristallin se composant principalement de chlorure de sodium, provenant de marais salants, de sel gemme ou de saumures provenant de la dissolution du sel gemme » [95]. Cette réglementation indique aussi que les sels doivent avoir au moins 97% d'extraits secs de chlorures de sodium (NaCl), additifs non inclus, et contenir moins de 2 mg/kg de cuivre ou de plomb, 0,5 mg/kg d'arsenic ou de cadmium et de 0,1 mg/kg de mercure.

Bien que l'iode soit, parfois, ajouté au sel afin de réduire les troubles thyroïdiens [96, 97], il est considéré comme un agent antimicrobien [98] et pourrait donc inhiber des microorganismes souhaitables dans l'affinage des fromages. Ainsi, les sels raffinés, tels que les sels de table qui contiennent de l'iode, ne sont pas appropriés pour la fromagerie [99]. Cependant, il n'y a pas beaucoup d'informations dans la littérature sur les types de sels utilisés pour la fabrication du fromage. Une recherche sur des sites de vente d'ingrédients de fromagerie, dans différents pays, confirment l'utilisation de sels marins ou du sel Himalaya, exempt d'iode. Ces deux types de sel sont riches en oligo-éléments (minéraux) et peuvent apporter un goût salé moins prononcé, en comparaison avec les sels de table. Enfin, en ce qui concerne le sel fromager, il est essentiel de

savoir à quelle vitesse (ou avec quelle lenteur) il est absorbé et contribue au processus d'extraction du lactosérum, à la formation de la croûte ainsi qu'au développement de microorganismes (bénéfiques ou néfastes).

2.3.2. Le sel comme source de développement de communautés microbiennes

Comme mentionné précédemment, les producteurs d'aliments utilisent le sel pour apporter des caractéristiques sensorielles bénéfiques ainsi que pour la conservation des aliments. Récemment, Haastrup *et al.* (2018) ont révélé la présence d'un microbiote complexe dans la saumure de quatre fromageries danoises, y compris les genres *Chromahalobacter*, *Debaryomyces*, *Microbacterium*, *Psychrobacter*, *Tetragenococcus* et *Debaryomyces* [100]. Cette étude suggère que certaines bactéries et levures, présentes dans la surface des fromages, pourraient être introduites par le sel marin. Par conséquent, s'il existe des microorganismes dans le sel utilisé, ils pourraient se retrouver aussi dans le produit final. Mais pourrait-il y avoir des microorganismes viables dans le sel ?

Le *Boston Magazine* [101] a présenté, en 2013, au grand public (non scientifique), une étude réalisée par Ben Wolfe (un microbiologiste de Harvard) et Scott Jones (un chef de cuisine), qui a démontré que certains sels renferment un grand nombre de microorganismes. Dans cette étude, ils ont utilisé sept types différents de sels de mer, minimalement traités, incluant les marques *Celtic*, *Amagansett*, *Wellfleet*, *French Grey*, *DeLallo*, *Bob's Red Mill* et *Maine*, ainsi que des techniques de microbiologie classique avec étalement des échantillons sur des boîtes de Petri (milieu de culture inconnu). De cette expérience, il a été constaté que les sels qui ont été très peu traités - humides, avec des cristaux plus gros et de couleur gris clair - contenaient le plus grand nombre de cultures viables. La plupart de ces bactéries sont des protéobactéries (genres/ espèces non mentionnés), un groupe de bactéries que l'on trouve couramment dans les océans. Il est aussi intéressant de noter que chaque sel avait son propre mélange de microorganismes, comme illustré, [Figure 5](#). Parmi les sept sels, trois étaient plus raffinés, d'un blanc pur, et ne comportaient pas de microorganismes détectables.

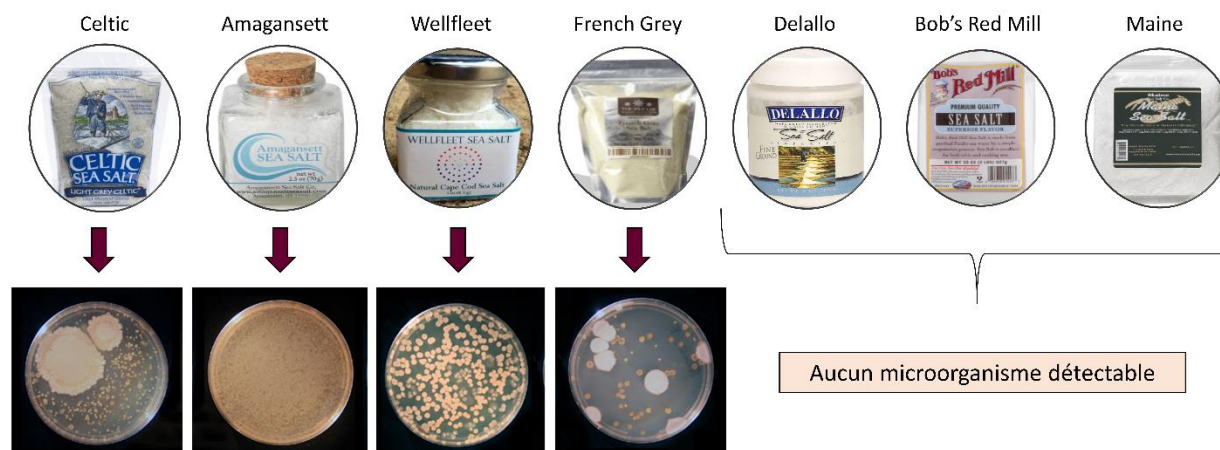


Figure 5. Les sept sels marins artisanaux analysés par Ben Wolfe and Scott Jones et leur pouvoir respectifs de détection de microorganismes (Boîtes de Petri avec milieu de culture inconnu) (Adapté de Boston Magazine [101]).

Une série d'isolats de sels commerciaux (ou alimentaires) a été obtenue au cours des dernières années, concernant principalement le groupe des Archaea (plus de détails dans le chapitre suivant, [section 3.1](#), [Fig. 8](#)). Cependant, il existe peu d'études indépendantes de la culture, dans la littérature, ce qui fait que le microbiote complet des sels alimentaires est encore faiblement exploré [55, 102]. Henriet *et al.* (2014) ont évalué la présence et la diversité des archées, par des méthodes de culture dépendantes et indépendantes, dans 26 sels de qualité alimentaire produits dans le monde (Amérique, Europe, Afrique, Asie et Océanie) [55]. Des comptages viables (en utilisant quatre milieux de culture pour Archaea) ont été observés pour plus de la moitié des sels (14), et ces comptages étaient supérieurs à 10^5 UFC/g dans les sels provenant de l'île de Gozo en Malte, de *Sel de Guérande* en France et de *La Palma Flor de Sal*, en Espagne. L'analyse métagénomique (gène 16S ARNr pour Archaea) réalisé pour neuf sels a montré la présence de 21 à 27 genres différents, la majorité correspondant à des membres de la famille des *Halobacteriaceae*, soit *Haloarcula*, *Halobacterium* soit *Halorubrum*, genres les plus représentés. D'autre part, Gibtan *et al.* (2017) ont évalué, par métagénomique (gènes 16S ARNr), la diversité de bactéries et archées pour quatre types de sel alimentaire, provenant d'Éthiopie et de Corée, produits à partir de bassins, de plaines salées, de vasières et de matériaux synthétiques [102]. Les résultats ont indiqué que les communautés microbiennes étaient très variables entre les sels évalués et contenaient environ 48-61% de bactéries et 38-51% d'archées. Parmi le groupe des Archaea, 91,6% appartenaient à la classe des halobactéries et les genres *Halarchaeum*, *Haloarcula*, *Halobacterium*, *Halonotius*, *Halomicrobium*, *Halorubrum*, *Natromonas* et *Salarchaeum* ont été répertoriés dans tous les sels.

Parmi les bactéries, les communautés étaient plus variables et *Aeromonas*, *Arthrobacter*, *Brachybacterium*, *Cronobacter*, *Enterobacter*, *Halovibrio*, *Leclercia*, *Microbacterium*, *Planococcus*, *Psychroflexus*, *Salicola* et *Salinibacter* figurent parmi les genres détectés dans un ou plusieurs sels.

Ainsi, tous les microorganismes tolérants au processus de traitement et à la forte concentration en sel peuvent persister en tant que cellule dormante et, lorsqu'ils trouvent des conditions favorables, peuvent se développer. Dans le cas d'études susmentionnées, les microorganismes ont été « réveillés » dans des milieux de culture de laboratoire, mais il est possible qu'ils se manifestent également dans les aliments.

3. Halophiles et halotolérants

Encadré 3 : Halophiles et halotolérants : de l'environnement aux aliments

Les microorganismes peuvent être classés en fonction de la concentration de sel nécessaire à leur développement: (i) halophiles : ils ont besoin de sel pour se développer et (ii) halotolérants : ils sont capables de se développer en présence ou en l'absence de sel. Des produits tels que les conserves de légumes, les fromages, les crustacés asiatiques et les viandes fermentées doivent une grande partie de leurs caractéristiques à l'action de microorganismes, dont la croissance donne forme à différents procédés technologiques, notamment l'ajout de sel.

Les microorganismes peuvent être classifiés en fonction de la concentration particulière de sel nécessaire à leur croissance optimale. Les groupes appartenant à cette définition sont appelés halotolérants, pour ceux qui tolèrent le NaCl, et halophiles, pour ceux qui ont besoin de ce composé pour leur prolifération [103, 104]. Il est difficile de définir ces termes, parce que leur classification dépend de plusieurs propriétés : la concentration minimale de sel requise pour la croissance des bactéries, la salinité optimale et la limite supérieure de sel tolérée dans l'environnement. De plus, ces paramètres dépendent souvent de la composition du milieu et de la température de croissance [105].

Les microorganismes halotolérants sont définis comme capables de survivre en présence ou en absence de sel [103]. Il existe plusieurs catégories d'halotolérants : (i) les non tolérants, ceux qui ne tolèrent qu'une faible concentration de sel (environ 1% p/v) ; (ii) les légèrement tolérants avec tolérance jusqu'à 8% (p/v) ; (iii) les modérément tolérants, de tolérance jusqu'à 20% (p/v) ; et (iv) les extrêmement tolérants, ceux qui se développent dans toute la gamme de concentrations de sel, comprises entre zéro à la saturation [103] ([Fig. 6A](#)). LeFevre et Round mentionnent, pour la première fois en 1919, la présence de microorganismes halotolérants isolés à partir de saumures de fermentation de concombres, à l'aide d'un milieu de gélose au jus de concombre avec rajout de 10% de sel [106].

D'un autre part, les bactéries aimant le sel, formellement appelés halophiles, sont des microorganismes qui ont besoin d'environnements salins pour survivre [103, 104]. La définition précise d'halophile diverge selon les auteurs, certains incluant tout organisme nécessitant des pourcentages autour de 3,5% de sel [103, 107], comme dans l'eau de mer, tandis que d'autres ne considèrent que ceux qui se développent de manière optimale à 5% ou plus, et tolèrent au moins 10% de sel [105]. Kushner, en 1978, a utilisé des définitions qui distinguaient différentes catégories : les halophiles légers (3% p/v de NaCl), les halophiles modérés (de 3 à 15% p/v) et les halophiles extrêmes (25% p/v) ([Fig. 6B](#)) [104].

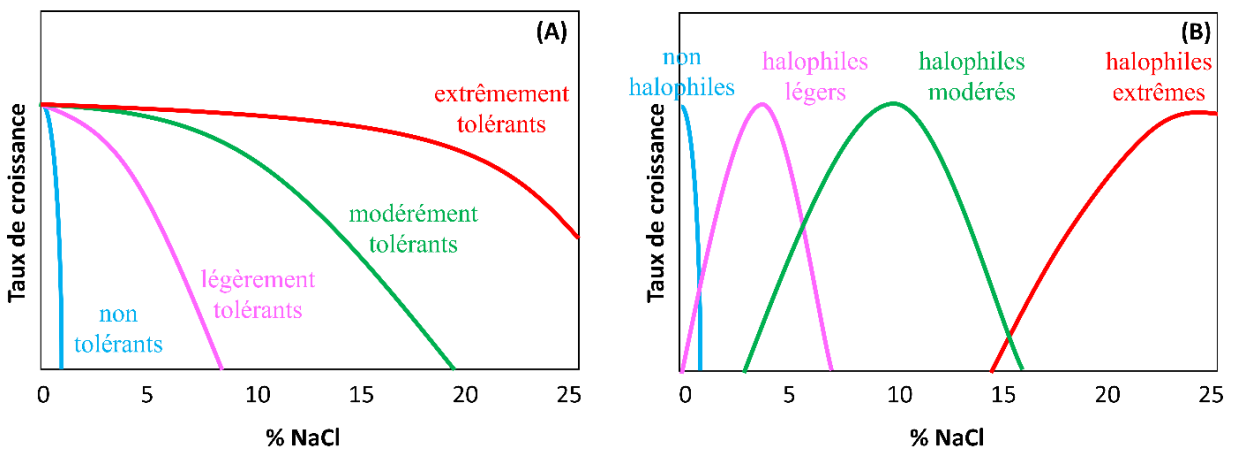


Figure 6. Classification des bactéries en fonction du pourcentage de NaCl nécessaire à leur croissance optimale. Halotolérants (A). Halophiles (B). (Adapté de Larsen [103]).

La vie, dans les environnements à forte concentration en sel, exige des stratégies pour équilibrer la pression osmotique cellulaire [107, 108], [Fig. 7](#). La stratégie utilisée par ces microorganismes consiste à maintenir de faibles concentrations de sel dans leur cytoplasme, en

équilibrant le potentiel osmotique avec des solutés compatibles [109]. Les solutés compatibles sont définis comme des osmolytes organiques (comme les polyols, les acides aminés et les amines quaternaires), qui peuvent être accumulés par la cellule à des concentrations extrêmement élevées sans perturber les fonctions cellulaires vitales. Par conséquent, cette composition peut contribuer de façon significative, en permettant, par exemple, de maintenir une pression osmotique interne plus grande que celle du milieu environnant afin de restaurer la turgescence cellulaire [110-112] (**Fig. 7B**). Pour s'adapter à un environnement très salin, les microorganismes peuvent aussi utiliser une autre stratégie appelée *salt-in* [111, 113]. Cette stratégie implique l'accumulation intracellulaire de concentrations molaires d'ions chlorure (Cl^-) et d'ions potassium (K^+), afin d'effectuer un ajustement thermodynamique de la cellule, augmentant la concentration en KCl (osmoprotecteur) dans le cytoplasme [105, 114] (**Fig. 7C**). Compte tenu de ces particularités, les halophiles et les halotolérants sont des microorganismes qui s'adaptent bien et peuvent jouer un rôle important dans leurs différents habitats.

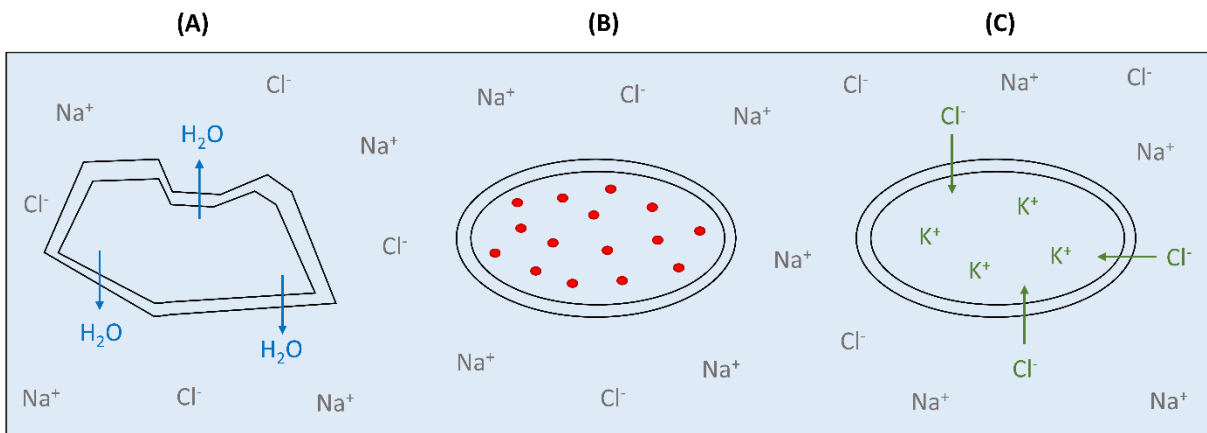


Figure 7. Les non-halophiles sont menacés dans les environnements salins, car ils perdent l'eau de leurs cellules (ce qui entraîne une plasmolyse) pour maintenir l'équilibre osmotique (A). Les halophiles utilisent des stratégies pour maintenir leur structure, soit par la synthèse de solutés organiques compatibles (B), soit par la concentration de K^+ dans leurs cellules (C).

3.1. Où résident les microorganismes halophiles?

Les microorganismes accompagnant le sel font partie des trois domaines de la vie: Archaea, Bacteria et Eucarya, et contiennent des représentants de nombreux types physiologiques, adaptés à une large gamme de concentrations de sel [115]. Une recherche des termes *halophil** en combinaison avec les mots *environment*, *food* et *cheese* - séparément - dans le titre, les mots clés et le résumé dans la base de données scientifique [Scopus](#) ont donné 2.498, 970 et 34 documents,

respectivement. Ce résultat souligne que ces microorganismes ont été principalement recherchés dans les écosystèmes naturels isolés des salines solaires artificielles, des lacs salés, des marais salants, des sols salins ou des mines de sel profondes.

En 2018, Loukas *et al.* [116] ont proposé une base de données halophiles appelée [HaloDom](#). Cette étude avait pour but de rechercher toutes les espèces halophiles documentées dans la littérature scientifique et d'intégrer ces informations sous la forme d'une base de données en ligne. Actuellement, HaloDom compte plus de 1.200 espèces halophiles (~475 genres), et englobe tous les domaines de la vie, dont environ 21,9% appartiennent à Archaea, 27,9% à Eukaryotes et 50,1% à Bacteria. Cette banque de données contient des informations de base telles que la taxonomie complète de chaque espèce, la zone de salinité où elles se développent, la classification selon le degré d'halotolérance (léger, modéré ou extrême), la disponibilité du génome et la bibliographie respective pour chaque espèce. Ainsi, les données accessibles à HaloDom ont été utilisées pour établir une phylogénie globale des microorganismes halophiles, au niveau du genre, et les mettre en relations avec leurs habitats. Pour cela, les genres qui n'avaient pas de génomes de référence séquencés dans la base de données du [NCBI](#) ont été filtrés (environ 40%). De plus, certains halophiles présents dans les aliments (et absents dans la base de données HaloDom), tels que *Brachybacterium*, *Marinomonas*, *Mucor*, *Pseudoalteromonas*, *Yarrowia* et *Trichoderma* ont été ajoutés à nos analyses, ce qui a conduit à un total de 276 genres halophiles (16 Eukarya, 55 Archaea and 205 Bacteria). Les principaux isolats de ces halophiles sélectionnés (ceux caractérisés par génomique ou décrits comme nouvelles espèces) ont été recherchés dans la littérature (surtout dans l'*International Journal of Systematic and Evolutionary Microbiology*), ainsi que dans les articles exploratoires ou de revue [55, 117, 118]. Ils ont alors été classés en fonction de leurs origines: (i) environnementale (sols, mer, lacs salins, mines de sel, etc), (ii) sel alimentaire, (iii) fruits de mer (principalement produits fermentés asiatiques), (iv) végétaux fermentés (olives, kimchi, algues salés, etc) ou (v) fromage/produits laitiers ([Fig. 8](#)).

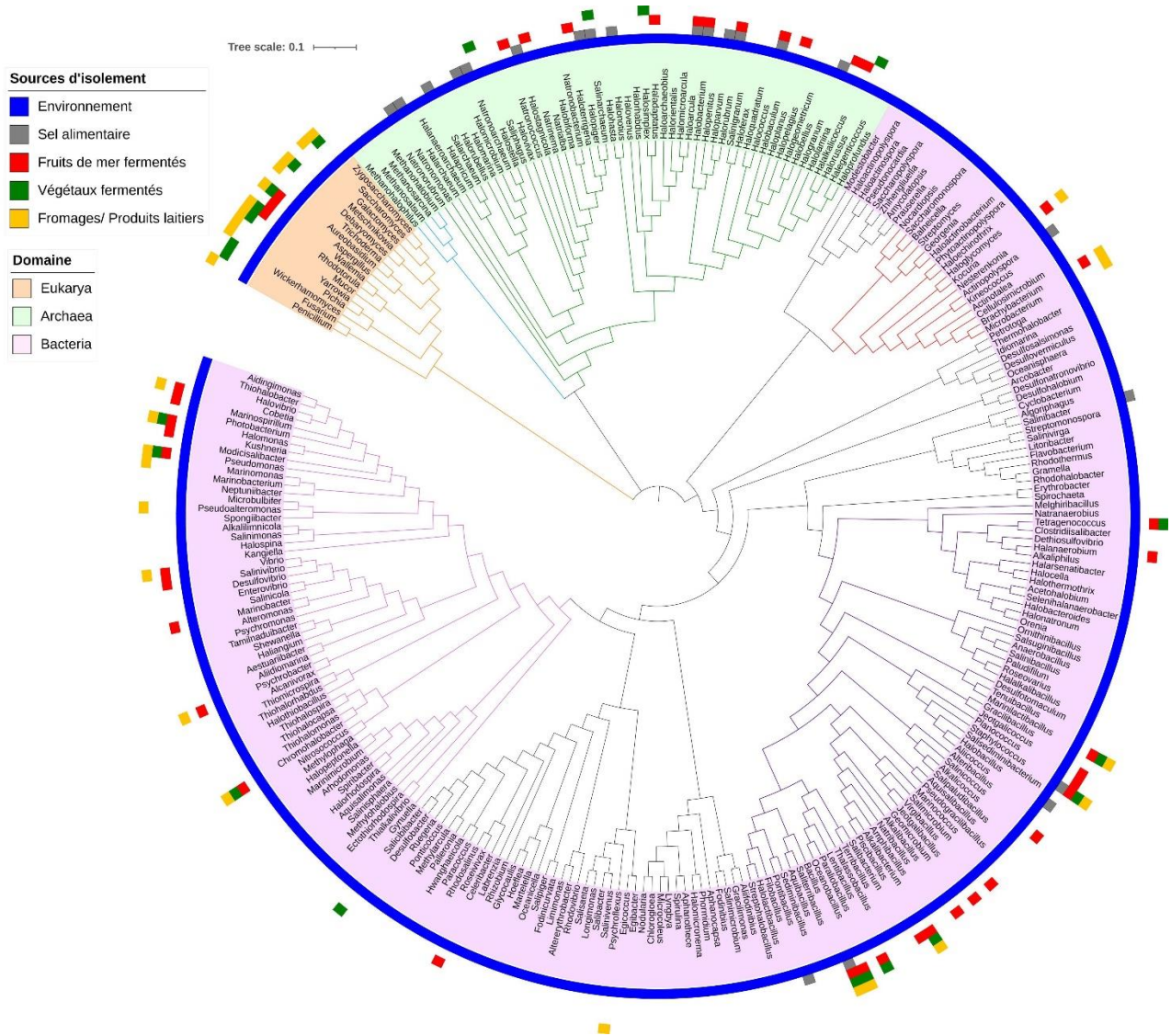


Figure 8. Arbre phylogénétique global des organismes halophiles. L'arbre a été construit par l'alignement du gène YchF comme marqueur, dans le logiciel MEGA 7.0.26 [119], en utilisant la méthode *neighbor-joining*, et finalisé avec *iTOL v5*. Les annotations des couches extérieures indiquent les sources des représentants présents pour chaque genre. Les zones orange, verte et rose clair soulignent, respectivement, les domaines Eukarya, Archaea et Bacteria, et les différentes couleurs des branches représentent les principaux groupes dans chaque domaine.

3.1.1 Eukarya

La Figure ci-dessus mentionne peu de représentants pour le domaine Eukarya, bien que la base de données HaloDom contienne 172 genres différents pour ce groupe. En effet, environ 90% d'entre eux appartiennent aux royaumes Animalia, Plantae et Protista, et leurs génomes complets sont absents dans la base de données publique du NCBI. Pour cette raison, ici, nous nous sommes concentrés sur le royaume des Fungi, qui comprend trois principaux phylums : Ascomycota,

Basidiomycota et Zygomycota [120]. Ces champignons halophiles ont été principalement isolés de la mer, des lacs salés et des biofilms microbiens [121]. Bien que souvent imperceptibles, ils sont présents dans tous les environnements de la Terre et peuvent être en interaction avec d'autres êtres vivants (archée, bactéries, etc.) dans les écosystèmes naturels ou alimentaires. Les espèces fréquemment rencontrées dans les aliments (particulièrement les fromages), incluent *Debaryomyces hansenii*, *Galactomyces candidum* et *Mucor* spp., ainsi que les moisissures *Penicillium roqueforti* et *P. camemberti*, qui sont ajoutées intentionnellement dans les fromages Roquefort et Camembert, respectivement [122]. Certaines espèces d'*Aspergillus* sont également présentes dans les produits alimentaires, mais sont généralement considérées comme contaminants et peuvent produire des aflatoxines qui sont des mycotoxines potentiellement dangereuses pour la santé humaine [123, 124]. En outre, *Rhodotorula glutinis*, une espèce du phylum Basidiomycota, peut être aussi considérée comme agent de détérioration dans les produits laitiers, légumes et fruits de mer [125, 126]. Cependant, cette espèce est une levure pigmentée particulièrement importante pour les industries alimentaires, en raison de son potentiel biotechnologique, notamment la production de caroténoïdes [126]. De plus, le genre *Mucor* regroupe de nombreuses espèces omniprésentes dans l'environnement (sol, air, poussière) [127], et peut aussi, régulièrement, se trouver, comme contaminant, dans la viande, les fruits, les légumes ou les produits laitiers ; leur croissance incontrôlée peut y induire des effets indésirables au niveau du goût, de la texture et de la coloration [128]. Cependant, plusieurs espèces de *Mucor* présentent également un intérêt technologique, agissant fortement sur la texture et les caractéristiques sensorielles de nombreux produits fermentés tels que les fromages [129, 130] ou le sufu (caillé d'haricots fermenté) [131]. Ainsi, certains représentants du royaume Fungi peuvent être bénéfiques pour les écosystèmes naturels et alimentaires et jouer un rôle écologique ou biotechnologique, mais ils peuvent aussi produire des effets néfastes ou indésirables.

3.1.2. Archaea

En ce qui concerne les halophiles du domaine Archaea, environ 90% des génomes complets sont disponibles (à partir de la base de données HaloDom), tous appartenant au phylum Euryarchaeota, et peuvent être répartis entre les classes Methanobacteria ou Halobacteria [132]. Les archées sont, en général, adaptées aux environnements extrêmes tels que les sources chaudes, les lacs salés ou les milieux très acides [133]. Celles qui produisent du méthane (méthanogènes)

ne sont rencontrées que dans les écosystèmes naturels comme les sols [134], les lacs salins [135] et les sédiments marins [136]. Au-delà des sources environnementales, la plupart des espèces archées halophiles sont aussi souvent isolées à partir de divers aliments salés, comme le sel alimentaire [102], les produits de mer salés et fermentés [137-139], la sauce de poisson [140-142] et les algues salées [143-146]. Henriet *et al.* (2014) ont montré que le sel non raffiné constitue un habitat favorable à de nombreuses et diverses espèces d'Archaea [55], dont la plupart ont été identifiées et appartiennent à la famille *Halobacteriaceae* (mentionnée précédemment dans la [section 2.3.2](#)). Ces organismes peuvent être « enfermés » dans des « inclusions fluides », qui sont de petites poches d'humidité qui se forment lorsque les cristaux de sel sont produits [147]. En effet, une étude, menée en 2010, montre que les genres *Halorubrum*, *Natronomonas* et *Haloterrigena* ont été piégés de cette manière dans des cristaux d'halite, en Californie (États-Unis), âgés de plus de 22.000 ans et ont pu être cultivés en laboratoire, en utilisant des milieux de culture et des conditions appropriés [148]. Concernant les aliments, Han-Seung (2013), dans un article de revue, a synthétisé la diversité des archées halophiles dans les produits fermentés [118]. En bref, les espèces des genres *Haladaptatus*, *Halalkalicoccus*, *Haloarcula*, *Halobacterium*, *Halorubrum*, *Haloterrigena*, *Natrinema* et *Natronococcus*, sont traditionnellement présentes dans les milieux naturels, mais on les retrouve aussi dans les poissons, les crevettes et des sauces salées orientales (en Corée et en Thaïlande). Ainsi, les archées ne sont pas seulement abondantes dans les environnements naturels, mais elles peuvent également se développer et survivre dans divers aliments à forte concentration en sel. Cependant, il n'y a pas de mention dans la littérature du domaine Archaea dans le fromage, possiblement parce qu'il est un produit contenant des quantités modérées de sel (environ 1-2%, [section 2.3](#)), valeurs insuffisantes pour développer une sélection nécessaire de ces organismes (communément) extrêmophiles.

3.1.3. Bacteria

Pour les bactéries, environ 90% des génomes sont accessibles via NCBI, en sachant que la banque de données utilisée (HaloDom) dispose d'une gamme plus large d'espèces pour ce groupe, en comparaison aux domaines Eukarya et Archaea. Comme pour les eucaryotes et pour les archées, les espèces bactériennes ont été, en général, isolées à partir dans différents écosystèmes naturels tels que le sol, les lacs et les mers [149]. Certaines souches halophiles environnementales sont des sources naturelles de biomolécules qui peuvent présenter un intérêt en tant que productrices de

pigments et d'activités antibactériennes [150, 151], ainsi que divers composés bioactifs, comme les antioxydants [152, 153]. Ces biomolécules présentent un intérêt commercial pour les industries alimentaires, pharmaceutiques, biomédicales et environnementales [154]. Relativement à l'isolement de bactéries dans les sels alimentaires, peu d'études sont mentionnées dans la littérature. Henriet *et al.* (2014), par exemple, ont isolé quelques espèces bactériennes originaires de sels en France (sels de Camargue et de Guérande) et à Malte (sel île de Gozo), comme *Actinopolyspora halophila*, *Halobacillus alkaliphilus*, *Pontibacillus marinus*, *Salinibacter ruber* et *Staphylococcus hominis* [55]. Concernant les isolats bactériens alimentaires, il est possible d'identifier leur présence parmi les différentes branches taxonomiques, en particulier dans les classes *Actinobacteria* (clade rouge), *Bacilli* (clade violet) et *Gammaproteobacteria* (clade rose) ([Fig. 8](#)).

Pour les *Actinobacteria*, plusieurs espèces de *Kocuria* et de *Brachybacterium* ont été isolées à partir de fruits de mer ou de produits laitiers. *Kocuria atrinae*, *K. koreensis* et *K. salsicia* sont des exemples d'espèces isolées à partir de *jeotgal* et *gajami-sikhae*, plats traditionnels coréens à base de crevettes, d'huîtres, de poissons et de céréales [155-157], tandis que *K. rhizophila*, *K. kristinae* et *K. rosea*, ont été isolées à partir du lait cru pour la production du Saint Nectaire, en France, et du fromage Parmigiano-Reggiano, en Italie [158, 159]. De la même manière, *Brachybacterium squillarum* a été isolé pour la première fois à partir de petites crevettes de Corée du Sud [160], alors que *Br. alimentarium* et le *Br. tyrofermentans* ont été isolés à la surface des fromages français, tels que le Gruyère et le Beaufort, et ont aussi été détectés, plus ou moins abondants, dans des croûtes de fromages à pâte molle, semi-dure et dure [3, 14, 30, 161]. Toujours dans le groupe des actinobactéries, l'espèce *Microbacterium gubbeenense*, décrite pour la première fois à partir d'un fromage à croûte lavée irlandais (Gubbeen) [162], a été retrouvée en grand nombre à la surface de divers fromages européens, tels que le Livarot, la Raclette et le Tilsit [30, 163], tandis que le *Microbacterium lacticum* est sporadiquement détecté dans les fromages à pâte dure ou semi-dure [164].

En ce qui concerne la classe *Bacilli*, plusieurs représentants de ce groupe sont détectés dans des poissons et des crevettes fermentées thaïlandaises (espèces de *Piscibacillus* [165], *Lentibacillus* [166] et *Tetragenococcus* [167]), ainsi que dans des fruits de mer fermentés traditionnels coréens (espèces de *Jeotgalicoccus* [168], *Salinicoccus* [169], *Planococcus* [170], *Virgibacillus* [171] et *Halanaerobium* [172]). Diverses espèces d'*Oceanobacillus* ont été aussi

détectées dans des aliments fermentés (à base de légumes ou de fruits de mer) en Corée, au Japon et en Thaïlande [173-176], tandis que ce genre est occasionnellement présent dans les produits laitiers [177]. En plus de cela, les bactéries lactiques halophiles et alcalophiles d'origine marine, comme les espèces *Marinilactibacillus psychrotolerans* et *Alkalibacterium* spp., peuvent contribuer à la flore bactérienne de diverses croûtes de fromages [39, 178-180], ainsi que de poissons, crevettes ou algues fermentées, au Japon et en Thaïlande [181, 182]. Parmi les genres bactériens, seulement deux, appartenant à la classe Bacilli, ont des représentants dans tous les habitats évalués (environnement, sel, aliments fermentés): *Bacillus* et *Staphylococcus* [183-186]. Ces deux genres comprennent des espèces pathogènes (comme *B. cereus* et *S. aureus*) [187] ou bien des espèces considérées comme bénéfiques (*B. coagulans* et *S. equorum*) [188, 189]. Certaines peuvent encore être utilisées pour les manipulations génétiques (en particulier *B. subtilis*) [190]. En bref, l'association de plusieurs espèces halophiles Gram-positives, membres des actinobactéries et bacilles, est bien étudiée comme ayant soit un rôle technologique bénéfique (qualité organoleptique, valeur nutritionnelle) soit nuisible (pathogène), dans les aliments tels que les produits laitiers, les végétaux fermentés, la viande et les produits de poisson [17, 191, 192].

D'une autre part, la classe des gamma-protéobactéries, qui comporte des espèces Gram-négatives, est largement étudiée dans les écosystèmes naturels en ce qui concerne ses applications industrielles et environnementales potentielles. Un grand succès de la biotechnologie est la production et l'application de l'ectoïne (principalement, grâce à *Halomonas elongata*) [193], un ingrédient actif dans de nombreux produits cosmétiques et de soins de la peau, qui devient de plus en plus important dans les préparations médicales [194]. Parallèlement, certains genres de ce groupe de bactéries peuvent être aussi isolés à partir d'aliments. Les espèces comme *Chromohalobacter*, *Pseudomonas* et *Halomonas*, par exemple, sont souvent présentes dans les sauces asiatiques de soja fermenté, les légumes et les fruits de mer [117, 195-199] ainsi que dans la saumure de fromages danoise [100] ou les croûtes de fromage [3, 40, 178, 200]. D'autres genres, communément présents dans les environnements marins, tels que *Cobetia*, *Psychrobacter*, *Marinomonas*, *Pseudoalteromonas* et *Vibrio*, sont sporadiquement isolés à partir d'aliments comme les fruits de mer et les végétaux fermentés traditionnels en Corée [201-204], et les fromages à croûte lavée [4, 205],

Bien que la plupart des halophiles soit connue pour vivre au sein d'écosystèmes naturels, nous avons identifié, ci-dessus ([Fig. 8](#)), la présence de certains représentants, régulièrement

détectée dans les produits alimentaires salés et fermentés, suggérant que les microorganismes des lacs, mer et sols peuvent s'adapter aux aliments. Cela soulève la question suivante : comment ces microorganismes environnementaux se sont-ils retrouvés dans les aliments ? Une première réponse possible, qui peut servir de base à notre compréhension de la répartition des halophiles dans différents habitats, peut être étayée par la célèbre loi de Baas Beeking [206] : « *Everything is everywhere: but, the environment selects* ». De plus, Selosse (2017) dans le livre « Jamais seul » rappelle que nous sommes entourés de multiples interactions mutuelles avec les microorganismes, que ce soit dans l'environnement, les animaux, les plantes ou même les aliments [207]. Ainsi, les microorganismes peuvent être répartis dans le monde entier et peuvent se manifester ou s'adapter à la niche alimentaire en gagnant ou en perdant des gènes, selon leurs besoins. Un paramètre potentiel pour la sélection de cette flore halophile dans les aliments est, particulièrement, l'ajout de sel.

3.2. Les protéobactéries dans les fromages

Encadré 4 : Impact technologique des halophiles dans les fromages

Certaines études considèrent que les bactéries halophiles tels que *Halomonas*, *Pseudoalteromonas* et *Psychrobacter* sont impliquées dans l'altération des aliments et sont considérées comme indésirables dans les environnements de transformation des aliments. Cependant, d'autres auteurs signalent que certaines espèces/souches produisent des quantités importantes de composés volatils, ce qui suggère qu'elles ont une fonction non négligeable dans l'affinage des fromages. Une gamme plus large d'espèces et de souches mérite d'être étudiée au cours du processus d'affinage du fromage et pourrait faire l'objet d'une étude plus approfondie afin de mieux comprendre la fonction concrète de ces gamma-protéobactéries dans les fromages.

Bien que les espèces halophiles de la classe Gammaproteobacteria aient été isolées de manière occasionnelle dans les aliments (et largement dans les écosystèmes naturels, comme illustré, [Fig. 8](#)), des études récentes, basées sur des méthodes indépendantes de la culture ont montré que ces bactéries peuvent être le microbiote dominant dans les surfaces de certains

fromages [3, 4], suggérant que leur rôle a été sous-estimé jusqu'à présent. Parmi les membres mentionnés ci-dessus ([section 3.1.3](#)), les *Pseudomonas* spp. sont les plus fréquemment impliqués dans l'altération des aliments et sont particulièrement importants dans les aliments réfrigérés, car certaines souches sont psychrotolérantes [208]. De nombreuses espèces de ce genre ont déjà été détectées dans les fromages, telles que *P. pseudoalcaligenes*, *P. alaligenes*, *P. aeruginosa*, *P. fluorescens*, *P. lundensis*, *P. brenneri*, *P. graminis* et *P. putida* [209-211]. La plupart d'entre elles sont déjà bien étudiées pour entraîner une altération des produits laitiers, due à la production d'enzymes extracellulaires thermostables et d'enzymes lipolytiques [212], qui peuvent également nuire à la qualité du fromage, en provoquant des saveurs amères ou un goût de rance (déjà mentionnées dans la [section 1.2.2](#)).

Si les bactéries Gram-négatives sont généralement considérées comme des indicateurs d'hygiène et d'altération des aliments, des études sur certaines d'entre elles ont montré que leur présence peut avoir un impact écologique et sensoriel, contribuant de manière significative à la qualité organoleptique du fromage [159, 213]. C'est le cas des genres marins *Halomonas*, *Pseudoalteromonas*, *Psychrobacter* et *Vibrio*, qui ont été régulièrement identifiés dans les croûtes de fromage [4, 5] et dans les surfaces des chambres d'affinage (seau de lavage, table de drainage, cuve de saumure, grille et drain) d'installations artisanales de fabrication du fromage aux États-Unis [7], ainsi que dans les équipements d'une fromagerie italienne [200]. Certains auteurs signalent que plusieurs espèces de ces genres produisent des quantités importantes de composés volatils tels que les sulfures, l'acétone, l'ammoniac et l'éthanol, ce qui suggère qu'elles participent à la production d'arômes [214-216]. Cependant, d'autres études considèrent que ces gamma-proteobactéries halophiles sont impliquées dans l'altération des aliments et sont, donc, considérées comme indésirables dans les environnements de transformation alimentaire [217-219].

La présence d'*Halomonas*, par exemple, dans les produits laitiers, a fait l'objet de discussions controversées. Quelques auteurs ont suggéré la présence d'*Halomonas* spp. comme indicateur des carences hygiéniques dans les installations productrices de fromage [178], pendant que d'autres études ont abouti au résultat indiquant que certaines espèces (*H. venusta/alkaliphila/hydrothermalis*, *H. boliviensis* et *H. variabilis*) peuvent avoir une fonction importante, lors de l'affinage du fromage, en particulier des jeunes fromages, qui sont lavées à la saumure plusieurs fois [4, 220]. Toutefois, il n'y a toujours pas de consensus sur l'interaction d'*Halomonas* concernant le goût ou la texture des fromages. Les espèces du genre *Pseudoalteromonas*, telles

que *P. prydzensis* [48] et *P. haloplanktis/nigrifaciens* [221] ont été déjà détectées dans des fromages, avec parfois une forte présence (>30% d'abondance relative dans une croûte de Munster [48]). Il a été récemment établi que certains représentants de *Pseudoalteromonas*, obtenus à partir de métagénomés de fromages à croûte lavée de la France et des États-Unis, peuvent avoir une activité lipolytique et protéolytique, contribuant aux propriétés aromatiques du fromage [3]. En plus, ces espèces (proches de *P. haloplanktis* TAC125) contiennent des lipases et protéases adaptées au froid, impliquées dans la production d'arôme, ainsi que le gène MGL (méthionine gamma lyase) qui transforme la méthionine en méthanthiol, un composé soufré [3]. Enfin, les espèces de *Psychrobacter* tels que *P. celer*, *P. cibarius/immobilis*, *P. faecalis* et *P. namhaensis* sont fréquemment rencontrées dans des échantillons de lait et de fromage [163, 221, 222]. Les membres de ce genre, en plus de tolérer le sel, sont psychrophiles et s'adaptent bien à l'environnement laitier. Des études ont montré que les souches fromagères *P. celer* 91 et *Psychrobacter sp.* 580 (espèce inconnue), contribuent, de manière significative, à l'affinage du fromage, car ils produisent des aldéhydes, des esters, des cétones et des composés soufrés [36, 214]. Ces souches ont été testées soit dans un modèle de fromage de croûte lavée, en interaction avec sept autres bactéries et quatre levures (souvent trouvées dans ce type de fromage), avec un affinage à 12°C, pendant 35 jours (souche 91) [36], soit dans un milieu liquide stérile, à base de caillé de fromage et sel, en association avec la levure *Debaryomyces hansenii*, et incubé à 14°C, en conditions aérobies pendant 41 jours [214]. Les espèces de ces trois genres (*Halomonas*, *Pseudoalteromonas* et *Psychrobacter*), se retrouvent généralement ensemble dans les fromages à croûte lavée, tels que le Livarot, l'Époisses, le Langres et le Munster, indiquant qu'ils partagent la même niche écologique et, potentiellement, développent des interactions [48, 221].

D'autre part, le genre *Vibrio* suscite beaucoup de controverses, car il comporte trois espèces principales, *V. parahaemolyticus*, *V. cholerae* et *V. vulnificus*, qui provoquent des intoxications d'origine alimentaire, en particulier dans les préparations à base de poisson [223]. Cependant, le genre *Vibrio* présente une grande variabilité génomique [224] et, certaines espèces, telles que *V. casei* et *V. littoralis* ont été détectées sur les communautés fromagères et peuvent avoir une contribution à l'affinage [3, 4, 7, 47]. Peu de choses sont connues sur les fonctions technologiques de ces espèces dans l'alimentation, mais Sawabe *et al.* [225], en 2013, ont proposé différents clades pour le genre *Vibrio*, en sachant que ces espèces présentes dans les fromages appartiennent au

clade Rumoiensis, qui est phylogénétiquement distant des pathogènes (clades Harveyi, Cholerae et Vulnificus) [224].

En bref, nous avons encore beaucoup à apprendre sur le rôle de ces microorganismes halophiles dans les aliments fermentés qui utilisent le sel naturel comme ingrédient. Étant donnée la grande abondance des gamma-protéobactéries dans certaines croûtes de fromage, leur rôle potentiel dans la technologie, le goût, l'arôme et leurs interactions avec la flore qui les accompagne est d'un grand intérêt, non seulement sur le plan scientifique, mais aussi sur le plan industriel et pour les consommateurs. De nouvelles recherches sont donc nécessaires pour identifier, avec précision, leur potentiel fonctionnel dans chaque écosystème alimentaire et, en particulier, le fromage.

CHAPITRE II – RESULTATS

1. Les bactéries halophiles et halotolérantes dans les croûtes de fromages

1.1. Contexte

Comme démontré dans l'introduction bibliographique ([section 3.1](#), [Fig. 8](#)), les bactéries halophiles sont généralement considérées comme vivant dans les environnements naturels, bien qu'elles puissent également être présentes dans les aliments fermentés, tels que le fromage. En effet, elles sont à même de constituer une partie importante des communautés microbiennes qui se développent à la surface des fromages [3], dont l'étude est encore peu développée, à ce jour. Le sel, l'un des ingrédients ajoutés aux aliments pour améliorer le goût et empêcher la multiplication des agents pathogènes, peut provoquer de profondes modifications au sein du fromage et constituer un facteur clé dans le développement d'écosystèmes particuliers, dominés par les bactéries halophiles et/ou halotolérantes. Bien que plusieurs études, celles des laboratoires de Rachel Dutton ou Paul Cotter en particulier, aient mis en évidence l'abondance de ces bactéries dans les croûtes de fromage, en utilisant la métagénomique et la métagénétique [3, 47], aucune ne s'est concentrée réellement sur leur isolement systématique. Effectivement, une recherche dans les collections de cultures, au niveau national et international, a démontré l'absence d'isolats halophiles alimentaires. Compte tenu de leur abondance et de leur fréquence dans certains fromages, nous réalisons que l'étude de ces bactéries peut être un outil d'un grand intérêt, à la fois industriel et scientifique. Ainsi, ce travail vise à :

- isoler les bactéries halophiles de forme systématique à partir de 13 croûtes de fromage ;
- séquencer les isolats obtenus, en définissant de nouvelles espèces éventuelles ;
- estimer la prévalence des génomes halophiles, originaires de produits laitiers, sur les métagénomomes de croûtes de fromages.

Les résultats obtenus ont été présentés sous la forme d'un article scientifique, dans la revue *International Journal of Food Microbiology* (partie 1.2).

1.2. Publication 1

Unraveling the world of halophilic and halotolerant bacteria in cheese by combining cultural, genomic and metagenomic approaches

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Abstract

Halophilic and halotolerant bacteria are generally assumed to live in natural environments, although they may also be found in foods such as cheese and seafood. These salt-loving bacteria have been occasionally characterized in cheese, and studies on their ecological and technological functions are still scarce. We therefore selected 13 traditional cheeses to systematically characterize these microorganisms on their rinds via cultural, genomic and metagenomic methods. Using different salt-based media, we identified 35 strains with unique 16S rRNA and rpoB gene sequences, whose whole genome was sequenced. The most frequently isolated species are the halotolerant Gram-positive bacteria *Brevibacterium aurantiacum* (6) and *Staphylococcus equorum* (3), which are also frequently added as starters. Their genomic analyses confirm the high genetic diversity of *B. aurantiacum* and reveal the presence of two subspecies in *S. equorum*, as well as the genetic proximity of several cheese strains to bovine isolates. Additionally, we isolated 15 Gram-negative strains, potentially defining ten new species of halophilic cheese bacteria, in particular for the genera *Halomonas* and *Psychrobacter*. The use of these genomes as a reference to complement those existing in the databases allowed us to study the representativeness of 66 species of halophilic and halotolerant bacteria in 74 cheese rind metagenomes. The Gram-negative species are particularly abundant in a wide variety of cheeses with high moisture, such as washed-rind cheeses. Finally, analyses of co-occurrences reveal assemblies, including the frequent coexistence of several species of the same genus, forming moderately complex ecosystems with functional redundancies that probably ensure stable cheese development.

Keywords: salt, cheese surface, microbial diversity, halophile, ecology, microbiology.

1 Introduction

Salt frequently plays a central role in the production of fermented foods (i.e., pickles, sauerkraut, cheeses, Asian seafood, fermented meats, etc.), not only contributing to an extended shelf life, but also leading to the development of particular aroma, texture, nutritional and beneficial health properties, eventually becoming part of the cultural patrimony in many countries. Although high levels of salt totally inhibit the growth of most microorganisms, moderate levels create an inhospitable environment for the majority of pathogens and promote the growth of certain microorganisms in various food products. Whereas many studies have focused on lactic fermentation in recent decades, the interest of the salt-driven development of non-pathogens in fermented food is more recent and at its beginning stages.

Microorganisms that grow in the presence of salt may be subdivided into two categories: (i) halotolerants, which are able to grow in the presence or absence of salt; and (ii) halophiles, which require salt to develop [1,2]. The precise definition of a halophile diverges depending on the authors, some of whom include any organism that requires percentages of around 3.5% of salt, as in seawater [1,3], while others consider only those that grow optimally at 5% or above, and tolerate at least 10% of salt [4]. Such halophilic bacteria have been widely described and isolated in different natural ecosystems such as soil, salt lakes and seas, and are of interest as producers of pigments and antibacterial activities, as well as a variety of bioactive compounds [5,6]. Halophilic and halotolerant bacteria have also been isolated from foods such as salt meat, shrimp, fermented fish sauce, sea food, poultry and cheese [7-10]. Several strains found in these foods have already been characterized, but their functions are still unknown. Certain studies consider that halophilic bacteria are involved in food spoilage and are thus considered to be undesirable in food processing environments [8,9,11]. Other authors report that several species produce significant quantities of volatile compounds such as sulfides, acetone, ammonia and ethanol, suggesting that they have a potential function in aroma production [7,12].

Overall, studies on halophilic and halotolerant bacteria in food are still scarce. A comprehensive overview of all ecosystem agents is therefore necessary in order to understand their functions and manage their evolution in the process. Cheese is part of these ancient fermented

foods in which salt addition may lead to major changes in its processing. There are more than 1,000 distinct cheese types worldwide, with a variety of textures, appearances, aromas and flavors that can be attributed to the technological development of complex and specific microbial communities, as well as to local factors such as milk source and farming practices [13-15]. The salting process might thus be one of the factors that strongly influence the way a microbial community will develop in cheese. Salt can be added in different ways - in crystal form or in solution, by brining or applying brine directly to the curd, by rubbing, smearing or scraping the surface, with clear salt solutions or historical old brines - and each of these processes may be applied once or several times and at different time scales (Fig. 1). Indeed, recent studies based on non-cultivable methods have shown that halophilic bacteria may be the dominant microbiota on cheese rinds, suggesting that their role has been underestimated until now [16,17]. A better understanding of their origin and how these organisms evolve during the different stages of cheese development could be of major interest to determine the role of salt in cheese ecosystem organization.

We therefore decided to enlarge the data repository of these salt-tolerant and salt-dependant bacteria in cheese in order to determine and produce a precise overview of their presence in cheeses produced using different technologies. For this purpose, we used culture methods to isolate and identify - in a systematic way - halophilic and halotolerant bacteria in 13 artisanal cheese rinds produced by different salting processes. We then combined genomic and metagenomic approaches that revealed potential new species, a wide diversity of halophilic and halotolerant bacteria in cheese rinds, and the coexistence of these species in this food ecosystem.

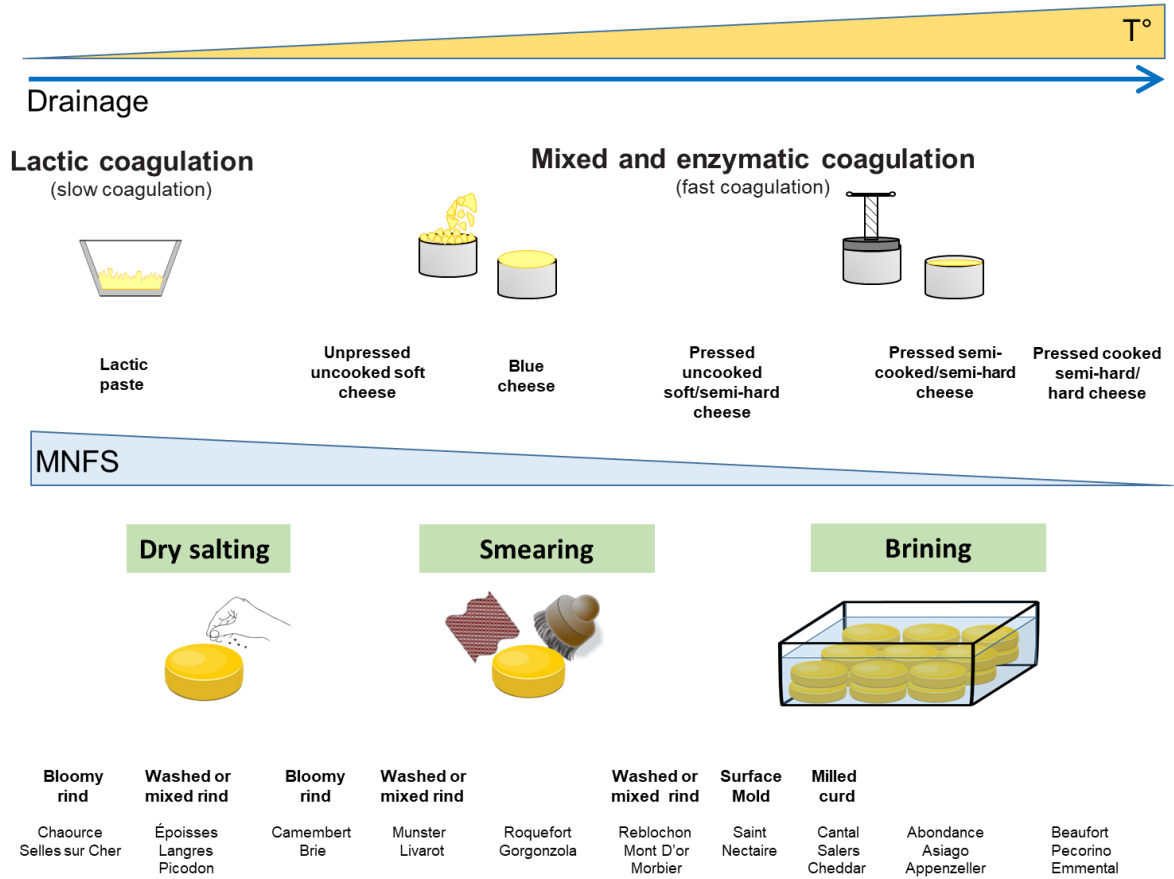


Figure 1. Cheese processing including examples of the salting process. Processes were classified as proposed by [55]. The effect of water activity depending on the process, in particular, on drainage and temperature, is primarily dependent on the moisture in non-fat substances (MNFS), whereas the salting level decreases this effect. Examples of cheeses are given to illustrate this figure.

2 Materials and methods

2.1 Cheese sampling

A total of 13 cheese samples were selected in this study (Table 1). The cheeses were purchased from a local supermarket and their rinds were sampled in portions (1 g) with a sterile knife and frozen at -20°C until further analysis.

Table 1. Metadata describing the 13 cheese rind samples.

Cheese	Cheese Technology	Animal	Rind Type	Origin (France)	Salting	Ripening*
A	Lactic paste	Cow	Washed	Bourgogne Champagne	Washed one to three times a week in brine	short
B	Lactic paste	Cow	Washed	Bourgogne Champagne	Washed one to three times a week in brine	short
C	Unpressed uncooked soft	Cow	Washed	Alsace-Lorraine	Washed three times a week in brine	short
D	Pressed uncooked soft	Cow	Washed	Savoie	Dry salt or brine before ripening	short
E	Pressed uncooked semihard	Ewe	Washed	Aquitane Midi- Pyrenées	Turned and brushed with dry salt or brine	medium
F	Lactic paste	Ewe	Washed	Méditerranée	Unkown	short
G	Blue cheese	Cow	Natural	Auvergne	Dry salt	medium
H	Pressed uncooked soft	Cow	Washed	Auvergne	Washed several times in brine and regularly returned	medium
I	Pressed uncooked soft	Cow	Natural	Franche-Comté	Dry salt	medium
J	Lactic paste	Goat	Bloomy	Méditerranée	Exclusively with dry salt	short
K	Pressed uncooked soft	Cow	Washed	Alsace-Lorraine	Washed twice a week in brine	medium
L	Pressed uncooked semihard	Ewe	Washed	Aquitane Midi- Pyrenées	Turned and brushed with dry salt or brine	medium
M	Pressed cooked hard	Cow	Washed	Franche-Comté	Turned and brushed with dry salt or brine	long

*short: several weeks; medium: several months; long: over 6 months

2.2 Enumeration and isolation of halophilic bacteria from cheeses

To enumerate and isolate halophilic strains, we used Marine Broth (MB; Difco, Sparks, USA), Long and Hammer Agar (LH; [18]) and *Halomonas* Medium (HM; [19]). Different concentrations of salt were supplemented in MB (0, 4, 6 and 8% NaCl). In order to prevent fungal growth, Amphotericin B (Sigma-Aldrich, St. Louis, MO, USA) was added to a final concentration of 20 µg/ml (50 mg/ml stock solution of Alfa Aesar™ Amphotericin B from *Streptomyces nodosus* in DMSO). Cultivable strains were enumerated using serial dilutions of homogenized cheese samples in sterile 0.9% NaCl solution. Population counts of cheese rinds were determined by 10⁻³ to 10⁻⁷ dilutions and incubated 48-72 h at 20, 25 and 30°C. For each cheese, the plates with a bacterial count comprised between 20 and 200 clones were selected for isolate characterization. An initial selection of apparently different isolates (morphotypes) was performed based on colony morphology (color, shape, elevation, pigmentation and opacity). A representative of each morphotype was then restreaked on a new plate for subsequent DNA extraction.

2.3 Identification of isolated morphotypes

The selected clones were collected with a sterile loop and mixed in a tube containing 300 μ l biomol water, 100 mg of 0.1 mm-diameter zirconium beads and 100 mg of 0.5 mm-diameter (Sigma, St. Louis, MO, USA). The tube was then vigorously shaken in a bead-beater (FastPrep-24, MP Biomedicals Europe, Illkirch, France) for 20 s at 4.5 m/s. The supernatant of this lysis was used directly for DNA amplification. The species assignment was performed by sequencing the 16S rRNA and the *rpoB* genes. The 16S rRNA gene was amplified using 27-F (5'-AGAGTTTGATCATGGCTCA-3') and 1492-R (5'-TACGGTTACCTTGTTACGACTT-3') [20]. The *rpoB* gene was amplified using primers VIC4 (5'-GGCGAAATGGCDGARAACCA-3') and VIC6 (5'-GARTCYTCGAAGTGGTAACC-3') [21,16].

Thermal cycling conditions applied for both were (i) 1 min at 94°C to initial denaturation; (ii) 30 cycles of 1 min at 94°C to denaturation, 0.5 min at 56°C to primer annealing, 1.5 min at 72°C to initial elongation; and (iii) 5 min at 72°C to final elongation. DNA amplifications were separated on 0.8% agarose gel. The PCR products were purified using the ExoSAP-IT (Thermo Fisher Scientific, Waltham, MA, USA) and sent for sequencing to the service provider (Eurofins Genomics, Ebersberg, Germany). Once received, the sequences were analyzed via the NCBI BLAST tool [22] to obtain a taxonomic classification for each isolate.

2.4 Genomic DNA extraction and sequencing

For each unique isolate, after cultivation in MB for 48 h at 25°C, DNA was extracted from the bacterial cells according to the protocol described by Almeida *et al.* [16] with some modifications. Briefly, we used an enzymatic lysis step followed by protein precipitation by adding potassium acetate. DNA was precipitated at -20°C after the addition of 0.1 volume of 3 M sodium acetate and two volumes of cold absolute ethanol to the upper phase. After centrifugation (30 min at 12,000 *g* and 4°C), the DNA was dried in a laboratory hood and resuspended in TE 1X buffer. The DNA concentration and quality was evaluated using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technology Inc., Wilmington, DE, USA). Additionally, 5 μ L of DNA were loaded on 0.8% agarose gel and visualized after migration by ethidium bromide staining.

DNA sequencing was carried out on an Illumina HiSeq at GATC-Biotech (Konstanz, Germany) in order to generate paired-end reads (150 bases in length). For each strain, the paired-end reads were merged and de novo assembly was performed using SPAdes, version 3.9 [23]. Only

contigs with length >300 bp and coverage >100 were considered for further study. Annotations were performed using the Rapid Annotation using Subsystem Technology server [24].

2.5 Phylogenetic analysis

For species assignation, evolutionary trees were built using the 16S rRNA and *rpoB* genes. To delineate species, we used a threshold of over 99% identity for 16S rRNA genes with type or well-defined strains [25], and of above 97.7% identity for *rpoB* nucleotide sequences [26]. Further phylogenetic analyses were performed using ClustalX 2.1 [27] and MEGA7 [28] and using the Neighbor-Joining method [29] with 1,000 bootstrap replicates [30]. Lastly, using genomic sequences, we determined the Average Nucleotide Identity (ANI) using JSpeciesWS [31] to confirm speciation of the different isolates. For new species delineation, we used the recommended cut-off point of 95% ANI [32].

2.6 Pan-genome of *Brevibacterium aurantiacum* and *Staphylococcus equorum*

ANI and pan-genome analyses were estimated for 25 genomes of *Brevibacterium aurantiacum* and 43 genomes of *Staphylococcus equorum* (Table S4). The ANI was performed using the ANIm method [33] and implemented in the Python module [PYANI version 0.2.6](#). The pan-genomes of both species were performed with Roary software version 3.11.2 [34] and the gene-based genome-wide association using Scoary [35]. Interactive visualization of genome phylogenies was done with Phandango version 1.3.0 [36].

2.7 Cheese DNA extraction and sequencing

From the 13 samples used to isolate halophilic and halotolerant bacteria, ten were selected to analyze their total DNA. The DNAs were prepared from cell pellets obtained from each cheese sample, following a method that combines enzymatic and mechanical treatments for cell lysis and treatment with phenol/chloroform/isoamyl alcohol to extract and purify DNA, as previously described [16]. The ten DNA samples were sequenced using Illumina HiSeq2500 technology at GATC-Biotech, which yielded 6-8 million paired-end reads of 150-nucleotide length. Moreover, 39 additional samples from different types of cheese were sequenced using SOLiD technology, which yielded 11-19 million single-reads of 50-nucleotide length. The raw read data for all samples are available under the accession numbers listed in [Table S5](#).

2.8 Quantification of species in metagenomic samples

First, species present in each metagenomic sample were identified with the Food-Microbiome Transfert tool, an in-house designed tool managing the following tasks. Each of the 66 reference genomes (one genome per species) was mapped on the metagenomic samples with Bowtie [37] (adapted to SOLiD data, parameters were adapted to take into account intra-species polymorphisms and choose at most one mapping position per read: first 35 nucleotides mapped; 3 mismatches allowed; --all --best --strata -M 1). In order to discard reads that could have been aligned on conserved regions on a more distant genome (same genus for example) or repeated regions, BEDtools [38] and SAMtools [39] were used to filter reads and compute genome coverage. Reads mapping genomic regions that were less informative and/or that could have been acquired by gene transfer (intergenic regions, tRNA, rRNA, genes annotated as “transposase”, “integrase”, “IS”, “phage/prophage” or “plasmids”) were not taken into account. In order to select genomes whose species is present in the sample, we selected genomes with at least 50% of their genes covered by at least one read. Food-Microbiome Transfert tool was used via a web interface developed via the Python Django framework as well as web technologies such as HTML and JavaScript. Genome, metagenome and analysis data are stored on a PostgreSQL relational database. Computations were performed on the Migale platform’s calculation cluster via the Bioblend API and the Galaxy portal.

Then, to determine the abundance of the different halophilic and halotolerant species, metagenomic reads were mapped on a database containing all 66 reference genomes with Bowtie (same parameters). We selected only reads mapping on genomes selected at the previous step. Quantification was done by counting the number of read for each genome. In order to obtain comparable results between metagenomes, we downsized the samples to 5 million reads. The metadata of metagenomic samples are presented in [Table S5](#).

2.9 Statistical analysis of co-occurrence relationships

The relationship between the halophilic species was examined by performing a correlation matrix using Pearson’s test. The function ‘rcorr’ (in ‘Hmisc’ package) was used to compute the significance levels ($p < 0.05$) and the graph were plotted using the ‘corrplot’ package for R. Only

species with a relative abundance $\geq 1\%$ were used to generate matrix and network correlations. Bacterial networks were explored and visualized using Gephi software 0.9.2 [40].

2.10 Data availability

Raw genomic reads were deposited to the European Nucleotide Archive under the project accession number PRJNA501839, while Illumina and SoliD metagenomic reads under PRJNA642396 and PRJEB39332, respectively.

3 Results

3.1 Abundance of halophilic and halotolerant bacteria in cheese rinds

For this study, we selected 13 artisanal cheese rinds whose main technological features are presented in [Table 1](#), in order to systematically characterize their halophilic and halotolerant microorganisms by culture methods. The bacterial population level was estimated by plate counts on several media and temperatures in order to optimize their growth and maximize the potential to isolate diverse strains. Overall, our results demonstrated the presence of dense bacterial populations (around 7-8 log CFU/g) on the surface of most cheese rind samples, with a minimum of 4.0 and a maximum of 9.5 log CFU/g ([Figure S1](#)). No major differences in bacterial counts were found between LH and MB media, whereas lower counts were obtained with HM medium for most cheeses. The effect of increasing salt was tested on MB medium and its increase negatively affected bacterial counts, in particular for Cheeses A, B and F. Interestingly, counts in MB+8% salt do not present major differences with those obtained in HM. The lower counts obtained in these two media, compared with other ones, could be due to a higher salt content: 8-10%, vs. 1 and 2% in LH and MB, respectively. Finally, the counts were similar at the three incubation temperatures tested (20, 25 and 30°C).

3.2 Defining potential new food halophilic species

Two marker genes, 16S rRNA and *rpoB*, were used to identify and differentiate the bacterial isolates. From ~320 isolates, we finally obtained 35 strains tentatively assigned to species, as described in [Additional File 1](#) and presented in [Table S1](#). Of these strains, 20 belong to the Gram-positive group and 15 to the Gram-negative group, 17 and three isolates of which are assigned at the species level, respectively. To obtain reliable taxonomic data, we determined the genomic

sequences of the 35 strains (Table S2) and performed an ANI analysis with closely related strains, as shown in Table S3.

Genomic analyses confirmed the assignment of the 17 Gram-positive bacteria carried out by the marker genes, while three remaining isolates belong to undescribed species (Table 2). *Brachybacterium* strain FME24 displays an ANI value of 83.17% with its closest relative *B. tyrofermentans*, indicating that it belongs to a new species. Similar analysis of *Brevibacterium* strains FME17 and FME37 showed that these two strains and *Brevibacterium* sp. 239c share an ANI of over 97% of each other (Table S3), but less than 87% with the closest reference species, indicating that they belong to a new species.

Concerning Gram-negative species, ANI analysis could assign only five isolates, while ten remained ambiguously or not assigned (Table 2). *Advenella* sp. FME57, isolated in this study, and *Advenella* sp. 3T5F (formerly referred to as *kashmirensis*) appear to belong to the same species (ANI=98.57%) but significantly differ from the *A. kashmirensis* type strain (WT001^T) with which they share an ANI < 90% (Table S3). These two strains should therefore belong to a new species. Concerning the *Halomonas* genus, ANI analysis showed that none of the strains isolated here could be assigned to an already described species, including the FME20 strain whose 16S rRNA and *rpoB* analyses suggested its assignment to *H. zhanjiangensis*. The ANI analysis demonstrated that the *Pseudoalteromonas* strain FME14 could be assigned to *P. prydzensis* (ANI=95.96%) and FME53 to *P. nigrifaciens* (best score ANI=98.31%) (Table 2). Furthermore, *Proteus* sp. FME41 and *Pseudomonas* sp. FME51 belongs to new species, since they share ANI<90% with their closest relatives (Table 2). Regarding the *Psychrobacter* genus, FME2 shares ANI~95% with five strains of *Psychrobacter*, including *P. immobilis* and *P. cibarius* type strains (Table S3), leaving its assignment unresolved. Finally, *Psychrobacter* strains FME5, FME6 and FME13 could not be assigned to any already known species by both markers and ANI analyses (Table S1 and Table S3).

Table 2. The 35 isolates and their cheese origin, media and temperature of isolation, as well as the closest species with their respective ANI values.

	Isolate in this study	Cheese	Media	Temperature isolated (°C)	Closest reference strains of whole-genome	ANI score (%)
Gram-positive strains	<i>Brachybacterium</i> sp. FME24	K	MB+6%NaCl	30	<i>Brachybacterium tyrofermentans</i> CNRZ926 ^T	83.17
	<i>Brachybacterium tyrofermentans</i> FME25	J	MB+8%NaCl	25	<i>Brachybacterium tyrofermentans</i> CNRZ926 ^T	95.89
	<i>Brevibacterium aurantiacum</i> FME34	G	MB+8%NaCl	20	<i>Brevibacterium aurantiacum</i> ATCC 9175 ^T	96.27
	<i>Brevibacterium aurantiacum</i> FME43	K	MB+8%NaCl	30	<i>Brevibacterium aurantiacum</i> ATCC 9175 ^T	96.29
	<i>Brevibacterium aurantiacum</i> FME45	K	HM	25	<i>Brevibacterium aurantiacum</i> ATCC 9175 ^T	96.62
	<i>Brevibacterium aurantiacum</i> FME48	C	HM	25	<i>Brevibacterium aurantiacum</i> ATCC 9175 ^T	96.67
	<i>Brevibacterium aurantiacum</i> FME49	L	MB+4%NaCl	20	<i>Brevibacterium aurantiacum</i> ATCC 9175 ^T	96.69
	<i>Brevibacterium aurantiacum</i> FME9	J	MB	25	<i>Brevibacterium aurantiacum</i> ATCC 9175 ^T	96.17
	<i>Brevibacterium</i> sp. FME17	M	MB+8%NaCl	20	<i>Brevibacterium aurantiacum</i> ATCC 9175 ^T	85.7
	<i>Brevibacterium</i> sp. FME37	K	MB	25	<i>Brevibacterium aurantiacum</i> ATCC 9175 ^T	86.18
	<i>Corynebacterium casei</i> FME59	L	MB+4%NaCl	25	<i>Corynebacterium casei</i> LMG S-19264 ^T	97.92
	<i>Glutamicibacter arilaitensis</i> FME22	H	MB	25	<i>Glutamicibacter arilaitensis</i> Re117 ^T	98.05
	<i>Carnobacterium mobile</i> FME4	A	MB	25	<i>Carnobacterium mobile</i> DSM 4848 ^T	97.42
	<i>Marinilactibacillus psychrotolerans</i> FME56	C	MB+4%NaCl	25	<i>Marinilactibacillus psychrotolerans</i> NBRC 100002 ^T	97.07
	<i>Oceanobacillus oncorhynchi</i> FME55	A	MB+8%NaCl	30	<i>Oceanobacillus oncorhynchi</i> Oc5	98.04
	<i>Staphylococcus equorum</i> FME18	H	MB	25	<i>Staphylococcus equorum</i> NCTC 12414 ^T	99.45
	<i>Staphylococcus equorum</i> FME19	G	MB+6%NaCl	25	<i>Staphylococcus equorum</i> NCTC 12414 ^T	94.85
	<i>Staphylococcus equorum</i> FME58	C	MB+8%NaCl	25	<i>Staphylococcus equorum</i> NCTC 12414 ^T	99.05
<i>Staphylococcus vitulinus</i> FME39	F	HM	25	<i>Staphylococcus vitulinus</i> DSM 15615 ^T	98.67	
<i>Staphylococcus succinus</i> FME10	D	MB	25	<i>Staphylococcus succinus</i> DSM 14617 ^T	97.77	
Gram-negative strains	<i>Advenella</i> sp. FME57	I	MB	25	<i>Advenella incenata</i> DSM 23814 ^T	90.42
	<i>Hafnia alvei</i> FME31	B	MB	25	<i>Hafnia alvei</i> ATCC 13337 ^T	99.58
	<i>Halomonas</i> sp. FME1	I	MB	25	<i>Halomonas boliviensis</i> LC1 ^T	79.97
	<i>Halomonas</i> sp. FME16	F	HM	25	<i>Halomonas zhanjiangensis</i> DSM 21076 ^T	82.23
	<i>Halomonas</i> sp. FME20	H	MB+4%NaCl	25	<i>Halomonas zhanjiangensis</i> DSM 21076 ^T	93.22
	<i>Proteus</i> sp. FME41	C	LH	20	<i>Proteus cibarius</i> JCM 30699 ^T	88.62
	<i>Pseudoalteromonas prydzensis</i> FME14	H	MB	25	<i>Pseudoalteromonas prydzensis</i> DSM 14232 ^T	95.96
	<i>Pseudoalteromonas nigrifaciens</i> FME53	B	MB+6%NaCl	25	<i>Pseudoalteromonas nigrifaciens</i> NCTC 10691 ^T	98.31
	<i>Pseudomonas lundensis</i> FME52	B	MB	25	<i>Pseudomonas lundensis</i> DSM6252 ^T	98.33
	<i>Pseudomonas</i> sp. FME51	C	LH	20	<i>Pseudomonas litoralis</i> 2SM5 ^T	87.7
	<i>Psychrobacter</i> sp. FME13	F	MB	25	<i>Psychrobacter fozi</i> CECT 5889 ^T	82.65
	<i>Psychrobacter</i> sp. FME2	A	MB	25	<i>Psychrobacter cibarius</i> JG-219 ^T	95.58
	<i>Psychrobacter</i> sp. FME5	G	MB	25	<i>Psychrobacter faecalis</i> Iso-46 ^T	80.7
	<i>Psychrobacter</i> sp. FME6	J	MB	25	<i>Psychrobacter faecalis</i> Iso-46 ^T	80.5
	<i>Vibrio casei</i> FME29	A	MB+4%NaCl	25	<i>Vibrio casei</i> DSM 22364 ^T	99.87

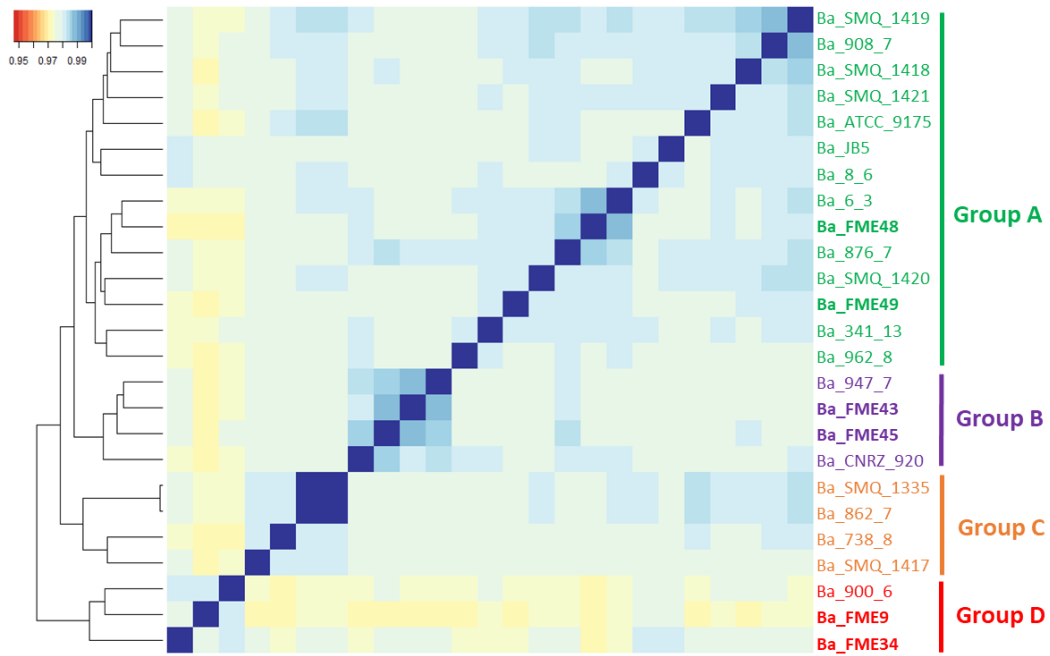
Therefore, from these 35 isolates, we obtained strains belonging to 26 different species, ten of which potentially belong to new species: two to the Gram-positive group and eight to the Gram-negative group.

3.3 Genomic diversity of *B. aurantiacum* and *S. equorum*

Among the Gram-positive bacteria, we isolated six *B. aurantiacum* strains, which, in addition to the 19 already sequenced genomes available in the NCBI database (Table S4), gave a total of 25 genomes. ANI analysis shows that they share over 97% identity and clustering analysis indicates the presence of four groups (indicated as A, B, C and D; Fig. 2A). The pan-genome analysis revealed an open pan-genome for 25 strains of *B. aurantiacum* with a total of 10,823 genes (Fig. S2A). We also analyzed the number of genes present in different numbers of k genomes, yielding two major groups. The first corresponds to the core genome (k = 25 genomes) and the second to the orphan genes (k = 1 genome), with 1,871, and 4,000 genes, respectively (Fig. S2B). Furthermore, we constructed the maximum likelihood tree from the accessory genome elements, which makes it possible to visualize the relatedness of strains based on their pan-genome composition and genes shared by different strains (Fig. 2B). This analysis indicates that strains belonging to groups B, C and D are also clustered together and shows that SMQ-1335 and 862_7 strains (isolated from cheese made in different regions) are very closely related, differing only by a few genes (Fig. 2B) and presenting an ANI of 99.94% (Fig. 2A). Moreover, FME43 and FME45 strains, both isolated from the same cheese in this study, belong to group B with an ANI of ~98.8%, while their gene content differs by about 10%. These strains are thus closely related but their pan-genome differs significantly.

In previous studies, potential horizontal gene transfer events were proposed to have occurred between *Brevibacterium* and several *Actinobacteria* [41-43]. From these studies, we selected ten regions containing genes involved in different metabolic functions and studied their distribution within the 25 *B. aurantiacum* genomes available (Fig. 2B). This analysis shows that, except for islands 3, 4 and 5, which are present in only one strain each, the other islands are widely spread out in the different *B. aurantiacum* strains. Concerning iron transport systems, which are carried out by islands 1 and 2, they are distributed in six and nine strains, respectively, and seem to exclude each other (Fig. 2B), probably to avoid the cost of their overload [44]. Eleven strains do not contain any of these additional genes, indicating that although they may confer a selective advantage, alternative systems exist.

(A)



(B)

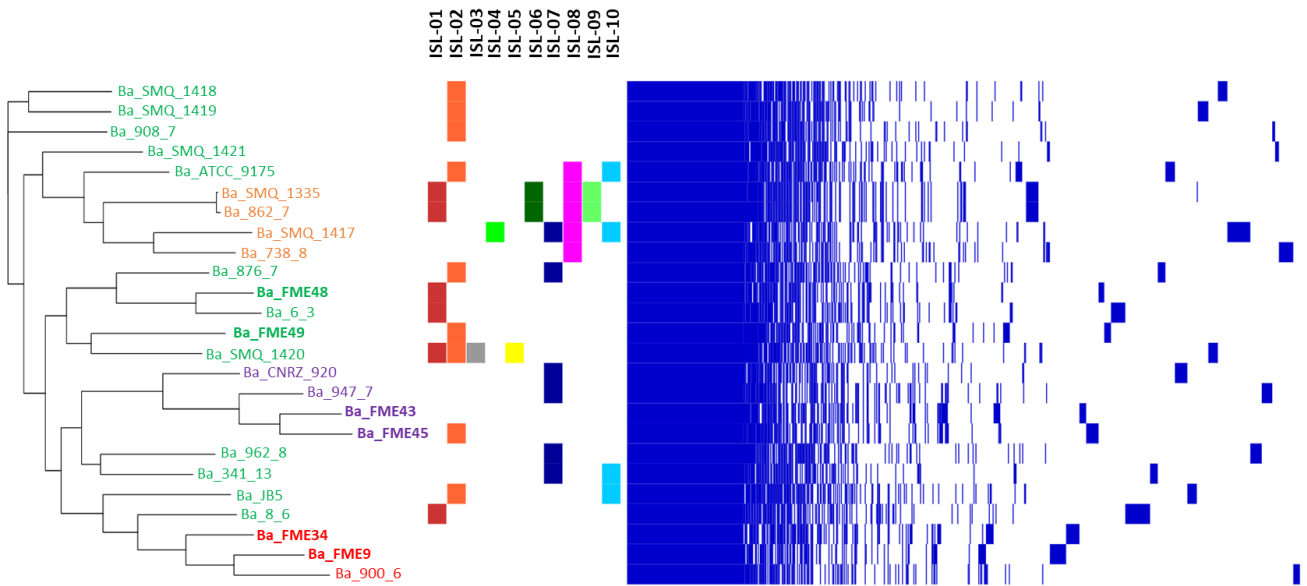


Figure 2. ANI and pan-genome analyses of 25 *B. aurantiacum* cheese strains. Strains from this study are highlighted in bold. (A) Phylogeny based on ANI values showing the presence of four groups marked in green (Group A), violet (Group B), orange (Group C) and red (Group D). (B) Pan-genome analysis. Left panel: maximum likelihood tree constructed from the accessory genome elements; middle panel: distribution of several horizontal gene transfer (HGT) regions described as islands [41]; Right panel: gene presence-absence matrix showing the presence (blue) and absence (white) of orthologous gene families.

Finally, we characterized three *S. equorum* strains, which, together with the other genomes of this species available in the NCBI database (Table S4), make a total of 43 strains. The tree based on ANI analysis revealed two well-separated groups of 39 and four strains, indicated as Groups I and II, respectively (Fig. 3A). Strains belonging to Groups I and II display an ANI > 98% within their group, but an ANI of ~95% with those of the other group. Group I, which is the largest, may be subdivided into three subgroups (A, B and C), sharing an average of 99.5% ANI intra-subgroups and differentiated by over 98.7% ANI inter-subgroups. While Group II contains only dairy strains, Group I also contains strains of cattle and several other environments (Fig. 3A). Pan-genome analysis of *S. equorum* indicated an open pan-genome with up to 7,000 genes (Fig. S2C). The analysis of the number of genes present in different numbers of k genomes revealed two major groups consisting of the core genome (k = 43 genomes) and the orphan genes (k = 1 genome), with 1,868, and 2,500 genes, respectively (Fig. S2D), which reflects a moderate level of genetic diversity in this species. Pan-genome clustering produced several groups, which were tentatively linked to metadata and ANI (Fig. 3B). First, it confirmed the distinction of Group II (FME19, White_SAM, OffWhite_SAM and BC9), whose strains mainly differ from each other by their content of mobile elements (prophages, potential plasmids, etc.), while the rest of their genome is nearly identical (2,292 genes > 99.9% identity). Furthermore, seven strains isolated in different cheeses and countries (France and the U.S.) appear to be highly related (908_10, Mu_2, 876_5, 862_5, 962_6, 947_12 and 738_7; Fig. 3B). They share ~2,500 almost identical proteins (> 99.7%), compared to ~1,400 with the other strains of this subgroup. Their pan-genomes mainly differ by mobile elements, including potential prophages, plasmids and the number of hypothetical proteins. Finally, while several cattle strains may also form distinct groups such as those of the ANI group B, several cheese and cattle strains appear to be related.

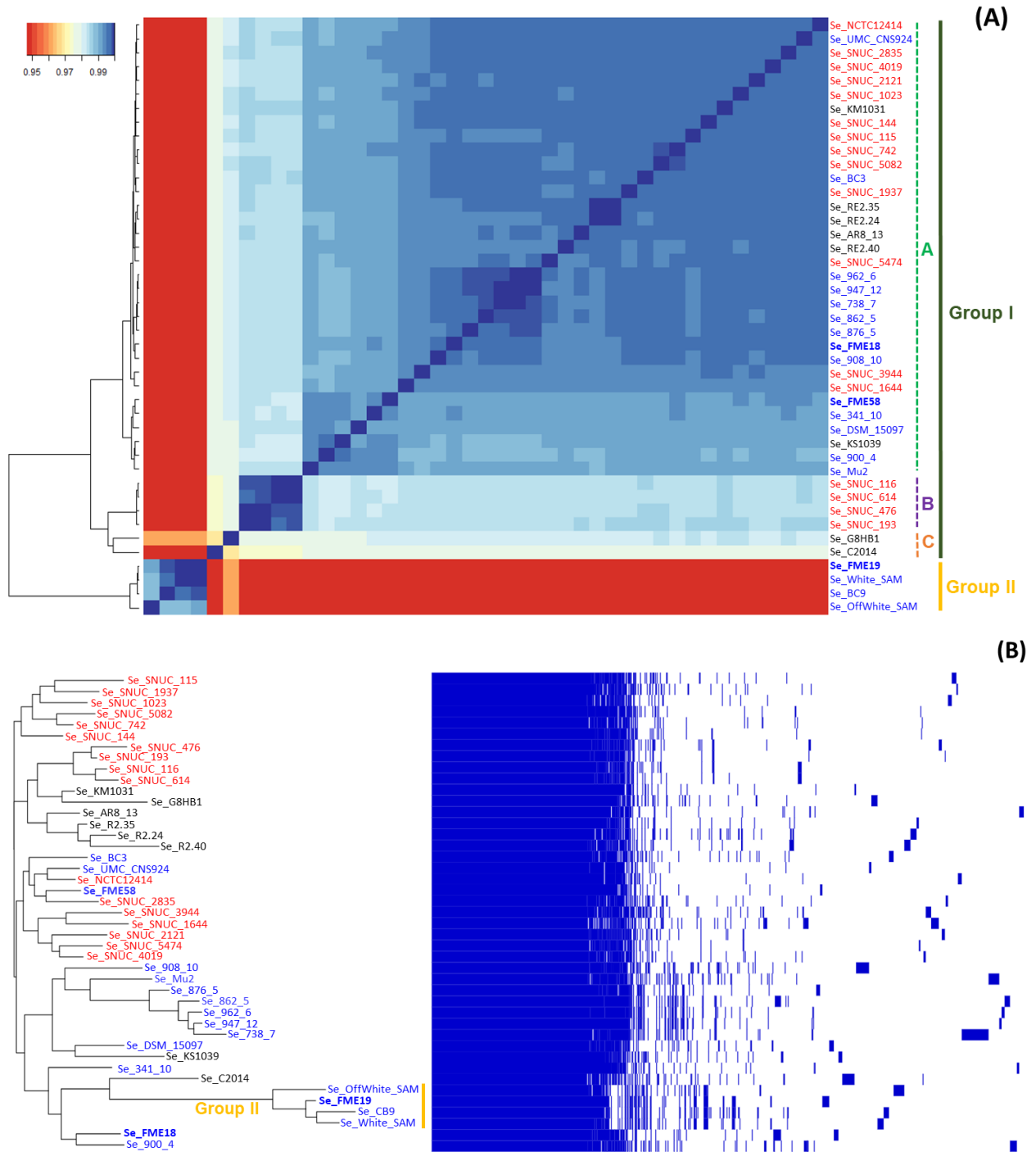


Figure 3. ANI and pan-genome analyses of 43 *S. equorum* strains. The origin of the strain is indicated by color: blue (dairy), red (cattle) and black (other). Strains from this study are highlighted in bold. **(A)** Phylogeny based on ANI values showing the presence of two groups potentially representing two subspecies; four subgroups in Group I (A, B, C and D) are presented by colored lines. **(B)** Pan-genome analysis. Left panel: maximum likelihood tree constructed from the accessory genome elements; right panel: gene presence-absence matrix showing the presence (blue) and absence (white) of orthologous gene families.

3.4 Overview of halophilic species in cheese rind metagenomes

The availability of the genomes sequenced in this study opens the possibility to detect and quantify their presence in shotgun metagenomic data. We therefore selected a set of 74 metagenomic samples corresponding to cheese rinds from different types, including 49 from this study and 25 from former studies [16,17] (Table S5). In order to provide a comprehensive overview of halophilic and halotolerant species in these samples, we completed our set with 40 supplementary genomes of related species isolated from cheese in previous studies. The percentages of reads corresponding to the 66 reference genomes, which were mapped on the 74 metagenomic samples, are presented in Table S6. Among the cheese rinds analyzed, only five have no detectable level of halophilic or halotolerant bacteria. Interestingly, more than half of the samples (42) present more than 10% of reads from these bacteria, showing their importance in cheeses.

Among Gram-positive bacteria, *Brevibacterium* and *Brachybacterium* species are widely distributed, especially in natural and washed cheese rinds (Fig. 4). *B. aurantiacum* is the most frequently detected, in about 70% of the samples, and its amount exceeds 10% in six cheese rinds. Interestingly, the new species of *Brevibacterium* isolated here, represented by FME37, is also frequently detected in cheese rind metagenomes (half of the samples) and exceeds 5% of the reads in three cheese rinds (Table S6). Similarly, the potential new species represented by *Brachybacterium* sp. FME24 is detected in 12% of the samples, showing the potential relevance of this species in cheese ecosystems. Both species of *Corynebacterium*, *G. arilaitensis*, *A. casei* and *M. gubbeenense* are also frequent (present in more than 17% of the samples) and sometimes abundant (more than 5% of the reads) in our dataset (Table S6). Additionally, coagulase-negative Staphylococci are particularly present in several natural cheese rinds (Fig. 4). Among the four species, *S. equorum* is the most frequent and abundant one.



Figure 4. Heatmap depicting the relative abundance (%) of halophilic and halotolerant species in 74 cheeses. Samples are ordered according to rind types, as indicated by upper labels (bloomy, natural and washed). Bacterial species are ordered according to their taxonomical class and whether they belong to the Gram-positive or Gram-negative groups, by the color of their name, black and blue, respectively.

Furthermore, several Gram-negative species appear to be frequent and dominant in a significant number of samples, especially in washed cheese rinds (Fig. 4). In particular, different species of *Halomonas*, *Pseudoalteromonas* and *Psychrobacter* are detected in 13 to 32% of the samples, and several species, including new species isolated in our study, exceed 10% of the reads (Table S6). Three additional Gram-negative species, *V. casei*, *V. litoralis* and *H. alvei*, are also relatively frequent (11 to 18% of the samples) and sometimes abundant (more than 5% of the reads mapped). Both *Vibrio* species are mainly found in washed rinds, whereas *H. alvei* is present in bloomy rinds (Fig. 4). Additionally, the *Pseudomonas* FME51-like species is only detected in the cheese sample it was isolated from, while *P. helleri* and *P. ludensis* are detected in seven and four samples, respectively, and sometimes at high levels (up to 25% of the reads, Table S6).

3.5 Co-occurrence relationships among bacterial species

Exploratory network and correlation analyses were performed to investigate the co-occurrence among cheese halophilic and halotolerant bacteria in order to identify combinations of species and ecosystem structuration (Fig. 5). As previously demonstrated (Fig. 4), Gram-positive species are more closely related in cheese with natural and washed rinds, while Gram-negative species are more closely related in cheese with washed rinds (Fig. 5A). Overall, the different species appear to present a higher level of co-occurrence within their group than outside (Fig. 5A and 5B; P value < 0.05). We observed that species belonging to the same genus are often found together, such as *Brevibacterium*, *Corynebacterium*, *Pseudoalteromonas*, *Halomonas*, *Psychrobacter*, *Pseudomonas* and *Vibrio* species (Fig. 5B). Additionally, we noted a positive correlation with *Brevibacterium* species, *Brachybacterium tyrofermentans* and *Agrococcus casei*. Finally, we highlight here the high level of co-occurrence between some species of *Psychrobacter* and *Vibrio*, as well between several species of *Halomonas* and *Pseudoalteromonas* (Fig. 5B; p value < 0.05).

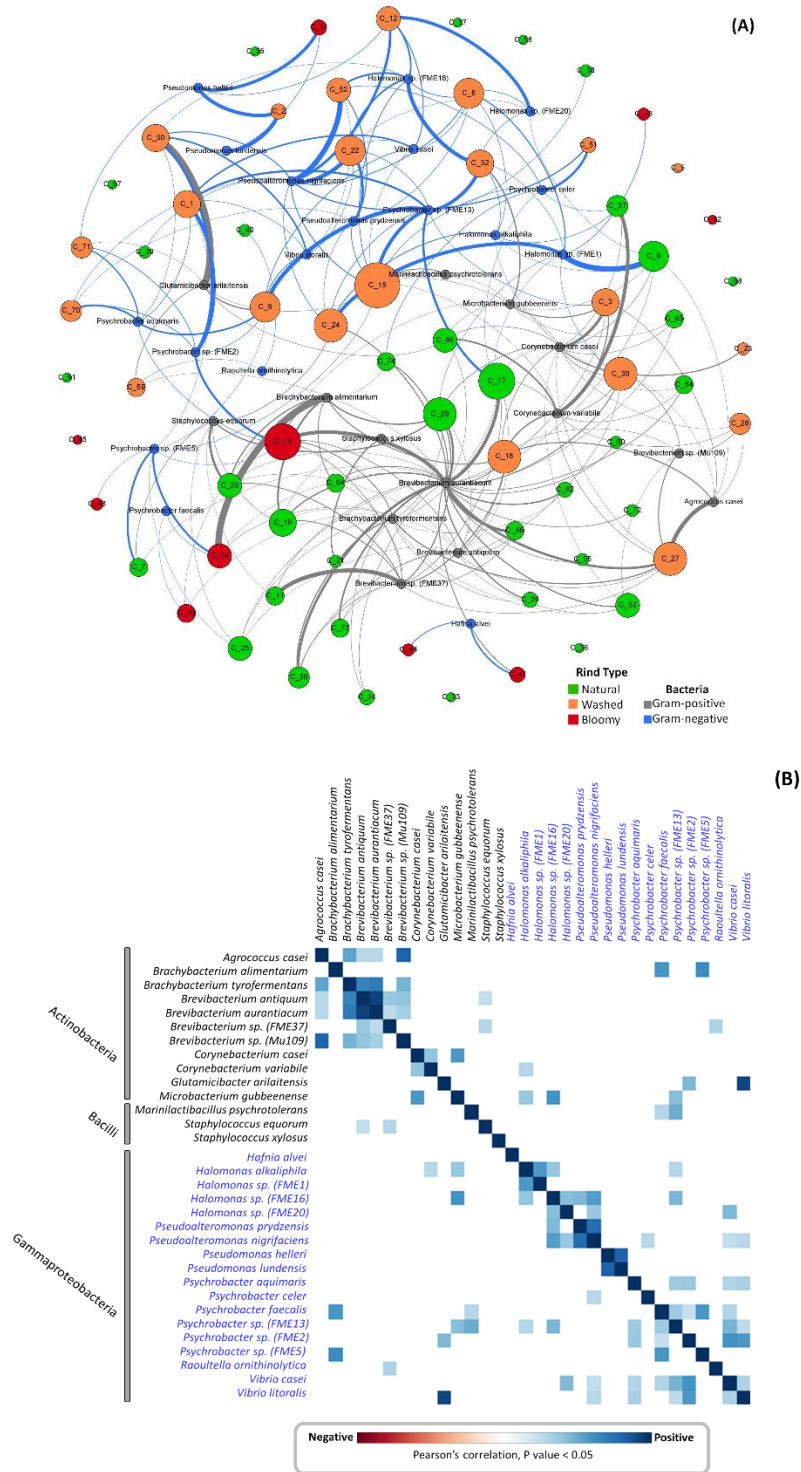


Figure 5. Relationships between halophilic and halotolerant bacteria detected by metagenomic analysis in cheese rinds. **(A)** Network summarizing the relationships between bacterial species and 74 cheese rind samples. For sample nodes, different colors (green, orange and red) are used to differentiate cheese rinds (natural, washed and bloomy, respectively). For species nodes, grey and blue are used to differentiate Gram-positive and Gram-negative strains, respectively. **(B)** Correlation matrix between halophilic and halotolerant species described in this study. Bacterial species are ordered according to their taxonomical class and whether they belong to the Gram-positive or Gram-negative groups, by the color of their name, black and blue, respectively.

4 Discussion

We isolated halophilic and halotolerant bacteria from the rinds of 13 traditional French cheeses by selecting colonies of different morphotypes on media containing 1 to 10% salt. Total halophilic population count was evaluated on media with three different basic compositions (HM, LH and MB), usually used in the study of environmental halophiles, and the effect of increased amounts of salt was tested on MB. Counts obtained with MB and LH media were similar, whereas they were lower on HM and MB+8% NaCl (Figure S1), possibly due to its higher salt concentration. In most rind samples, halophilic and halotolerant populations were 7-8 log CFU/g on the former media, which is a range similar to those reported in other studies using different culture media supplemented with salt, such as Milk Plate Count Agar (MPCA) with 5% salt [45,46], Trypticase Soy Agar (TSA) supplemented with 4% NaCl [47] and Brain Heart Infusion (BHI) [48]. These data, together with the fact that most isolates were able to grow on all tested salt-based media, would indicate that the choice of the media is not determinant in the study of halophilic and halotolerant bacteria in cheese. Nevertheless, we did not always obtain the same species in the parallel plate isolation, suggesting that the use of different media could favor, differently and sufficiently (without being sharply selective), the growth of different populations and thus allow a greater variety of species to be isolated. From ~320 isolates, we selected 35 strains with unique 16S-*rpoB* sequences corresponding to 20 Gram-positive and 15 Gram-negative strains (Table 2). Their genome sequences were determined and ANI analysis showed that 12 of these isolates belong to two and eight potentially new species of Gram-positive and Gram-negative bacteria, respectively.

4.1 Halophilic and halotolerant as food bacteria

The availability of the genomic sequences of halophilic and halotolerant cheese bacteria offers the opportunity to reliably detect and quantify their corresponding populations in cheese metagenomic samples at a level of 0.1% of DNA. This relative abundance level corresponds to subdominant populations, which may reach 10^5 - 10^6 CFU/g for cultivable species in several types of cheeses, a level compatible with a significant metabolic activity that could impact cheese technology.

The development of Gram-positive species belonging to *Actinobacteria* and *Firmicutes* in cheese rinds during ripening is well established and has been previously reported in different types of cheeses all over the world ([Additional File 2](#)). A metagenomic search showed that species like *B. tyrofermentans*, *B. aurantiacum*, *Corynebacterium* species and *G. airilaitensis* were detected in 25 to 70% of samples at maximal levels of 6 to 45% of the reads ([Table S6](#)), confirming their wide distribution in cheese rinds and their potential importance in cheese technology. Interestingly, we isolated a new species of *Brevibacterium* (FME37 strain) that is widely distributed in metagenomic samples and sometimes detected at high levels (>20%), suggesting a role in cheese.

B. aurantiacum is the most frequent and abundant *Actinobacteria* found in our metagenomic samples. This frequency was probably boosted by the over 40-year-old history of the use of this bacteria as an adjunct to take advantage of its various technological properties [49,50], although the persistence of adjunct strains is questioned [51,52]. This species was subject to the most detailed genomic analysis, and 19 cheese genome sequences were available at the time of this study. The comparison of these genomes, in addition to the six provided here, confirmed the substantial genetic diversity within *B. aurantiacum* and its plasticity, which might be reflected in the diversity of color, aroma, lipolytic and other technological factors described for this bacterium. Finally, the study of the different genomic islands – characterized earlier – suggests that their roles are not crucial for the development of this species in cheese since a significant number of strains are not concerned by these additional factors. In particular, from the two strains FME43 and FME45 (isolated from the same cheese), only one contains iron acquisition genes on ISL2 ([Fig. 2B](#)), which were described as being important to develop within the cheese surface habitat [42,53]. The alternative distribution of the two different iron acquisition systems on ISL1 and ISL2 in about half of the strains may suggest that their presence is also a metabolic load, whereas alternative strategies probably exist.

Furthermore, we isolated three coagulase-negative staphylococci species, a type of bacteria commonly isolated from cheese. In agreement with a previous metagenomic study [54], we found that *S. equorum* is the most frequent and abundant species, while *S. succinus* and *S. vitulinus* are more scarce ([Fig. 4](#)). *S. equorum*, which is also used as an adjunct in cheese to improve its texture and contribute particular flavors ([Additional File 2](#)), has been extensively studied for safety reasons, and 40 genomes from food, cattle and clinical samples were available at the time of this study. Their ANI analyses, together with our three isolates, suggest that *S. equorum* could be

subdivided into two subspecies (Figure 3A), the second one being represented by four cheese strains. The combined ANI and pan-genome analyses of these strains indicates that they mainly differ by their mobile element content (prophages and potential plasmids), whereas the rest of their genomes are nearly identical, suggesting a recent common origin for their use in cheese. Further investigations will be required to determine if the two potential subspecies express different technological properties, including phage resistance. In the Group IA, many isolates from cheese could not be clearly differentiated from those from cattle, suggesting their animal origin, except a group of seven cheese strains (Fig. 3B). The latter ones mainly differ by their mobile elements, which could be the result of starter culture selection. However, further studies will be necessary to demonstrate that these strains followed a drift and are now specifically growing in cheese rinds.

Additionally, we isolated three other less documented *Firmicutes*, including *Marinilactibacillus psychrotolerans*, *Carnobacterium mobile* and *Oceanobacillus oncorhynchi* (Additional File 2). Whereas *M. psychrotolerans* was detected in around 13% of our metagenomic data with a maximum of 4% of the reads, *C. mobile* and *O. oncorhynchi* were not detected, including the samples from which they were isolated (Table S6). These results indicate that the size of the population of these two species was very small and that they may not play a significant role in cheese technology.

In addition to these halotolerant Gram-positive bacteria, we obtained several Gram-negative species that have not yet aroused keen interest in technological developments. Remarkably, *Gammaproteobacteria* from the genera *Halomonas*, *Pseudoalteromonas* and *Psychrobacter* were the most frequently isolated (representing 60% of the Gram-negative isolates obtained in this study), and they often represent high relative abundance in washed cheese rinds (Fig. 4). This observation is in agreement with recent culture-independent analyses [16,17], and their presence in cheeses of good quality supports the possibility that they may have a positive impact during ripening, while several authors consider them as contaminants (Additional File 2). Nevertheless, the characterization of the potential role of these genera in cheese technology remains to be explored. Finally, we isolated species of *Pseudomonas*, *Proteus*, *Hafnia*, *Vibrio* and *Advenella*. All these genera have already been reported in cheese rind communities and the abundance of several of these species may support further interest for their role in cheese ripening (Additional File 2). Currently, *Hafnia alvei* is the only Gram-negative bacterium used as a ripening starter in

the cheese-making process and it was detected here at level of 4.5 and 8.5% in two bloomy cheese rinds (Table S6), where it was probably added as an adjunct culture.

4.2 New insights into cheese microbial ecology

Interestingly, the development of Gram-negative bacteria appears to be greater in soft cheese, and also favored by the washing and smearing processes of the rind, which correspond to cheeses displaying higher moisture [55], as roughly depicted in Figure 1.

Remarkably, the present metagenomic analysis discriminates populations at the level of species, which is not always possible with amplicon sequencing that is limited by the lack of significant divergence, especially for Gram-negative species such as those presented in this study. Consequently, the present set of data uncovers the world of halophilic and halotolerant bacteria in cheese rinds with a high level of resolution, and reveals, in particular, the co-occurrence of a number of species. For example, species of *Brevibacterium*, *Corynebacterium*, *Halomonas*, *Psychrobacter*, *Pseudoalteromonas* and *Vibrio* are found co-occurring with at least another species of the same genus (Fig. 5B). The coexistence of such a variety of species reflects the fact that these ecosystems are open to the microbial environment, which is likely to be resilient to their production conditions and develop with salt as a key driver. The presence of related species, which are thus likely to carry out similar metabolic functions, will lead to a functional redundancy, a factor that was proposed to be of primary importance in the resilience of ecosystems submitted to changes or pressures [56,57]. In the context of traditional productions, changes could encompass modification of milk quality, technological issues and phage attacks, thus structuring the ecosystem and maintaining a variety of microorganisms. However, while the presence of diverse microorganisms in the processing environment may increase the ecosystem's capacity to respond to changes, it may also modify the organoleptic properties of cheeses [58]. Further studies will be required to understand the interactions that occur between these microorganisms and their role in the development of cheese, as well as the development of the rich and various organoleptic properties of traditional products.

In conclusion, salt is commonly added to food to prevent the growth of pathogens by decreasing water activity, which leads to profound changes in the environment and leads to the development of particular ecosystems dominated by halophilic and halotolerant bacteria. This is the case of cheese, where these communities have not been thoroughly explored and whose origins

are probably multiple. Using a combined approach that includes cultures, genomics and metagenomics, we have identified ten potential new species that are widely distributed in 74 cheese rind samples. Co-occurrence and distribution network analysis indicates that phylogenetically related species coexist and probably together contribute to a safe and stable development of cheese. The isolation of new species that we have used here as references to advance the understanding of these ecosystems, will also allow functional studies of this particular world structured by the addition of salt.

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Authors contributions

PR conceived the study and its experimental design. BD and BFK collected samples, isolated and identified strains. CIK performed genomic and metagenomic analysis. AB contributed to genomic analysis. The Food Microbiome team supported overall cheese rind metagenomic analysis. CIK and PR analyzed the data and wrote the manuscript. PR supervised the project.

Competing interests

The authors declare that they have no competing interests.

Supplementary Material

The supplementary Table S1-S6 and the Additional Files 1 and 2 can be found at <http://dx.doi.org/10.17632/7vc3vndc3r.1>.

Additional File 1. This supplementary information documents the identification of halophilic and halotolerant isolates using the phylogenetic analysis of 16S rRNA and *rpoB* genes.

Among the 20 Gram-positive bacteria isolated here, twelve belongs to Actinobacteria and eight to Bacilli class (**Table S1**). The Actinobacteria were distributed in four genera: *Brachybacterium* (2), *Brevibacterium* (8), *Corynebacterium* (1) and *Glutamicibacter* (1). Among these strains, nine could be assigned unambiguously at the level of species to yield *Brachybacterium tyrofermentans* (1), *Brevibacterium aurantiacum* (6), *Corynebacterium casei* (1) and *Glutamicibacter arilaitensis* (1). However, the second *Brachybacterium* isolated in this study (FME24) could not be assigned reliably to a species although its 16S rRNA share around 99% identity with the to *B. tyrofermentans* type strain, since its *rpoB* nucleotide sequence only shares 93.43% with this strain (**Table S1, Fig. A**).

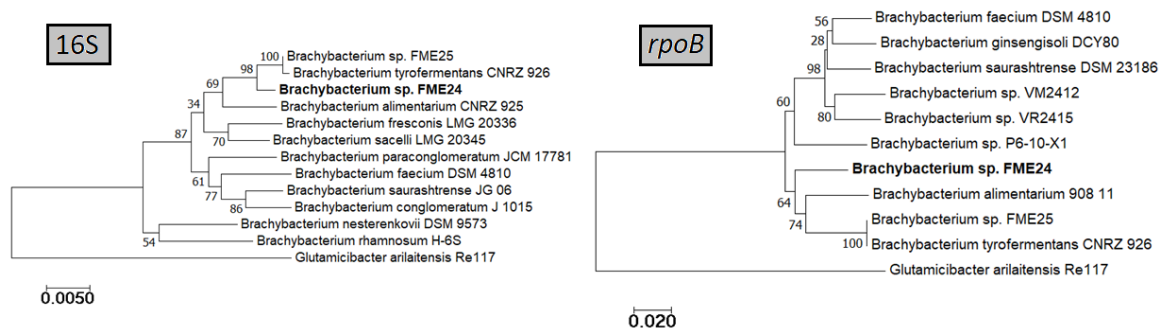


Figure A. Phylogenetic tree of *Brachybacterium* genus based on 16S rRNA and *rpoB* gene alignments, including FME24 and FME25 strains (isolated in this study) and their closest relatives.

Beside, the two last *Brevibacterium* (FME17 and FME37) 16S rRNA displayed only ~98% identity with the type strain of *B. antiquum*, a level which is insufficient to assign them reliably at this species. Moreover, their assignation to this species could not be confirmed by the analysis of their *rpoB* sequences, which share less than 96% identity (**Table S1, Figure B**).

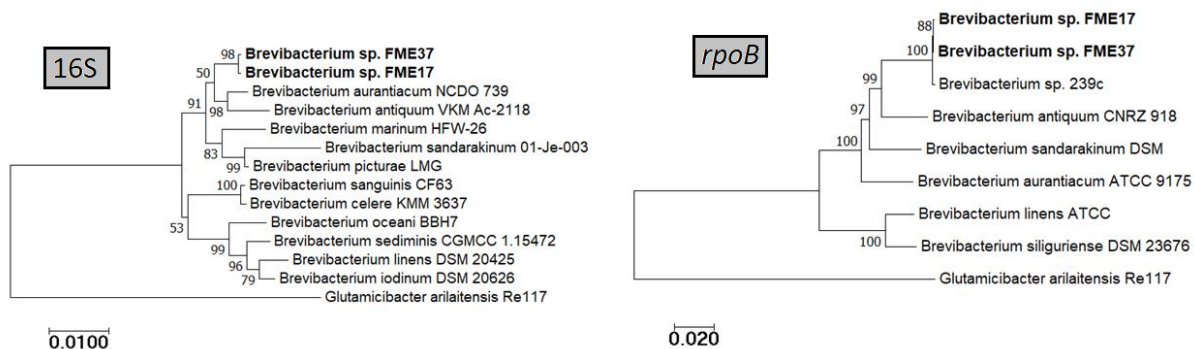


Figure B. Phylogenetic tree of *Brevibacterium* genus based on 16S rRNA and *rpoB* gene alignments, including FME17 and FME37 strains (isolated in this study) and their closest relatives.

Finally, isolates belonging to Bacilli class were distributed in four genera that could be unambiguously assigned at the level of species as follows, *Carnobacterium mobile* (1), *Marinilactibacillus psychrotolerans* (1), *Oceanobacillus orcorhynchi* (1), *Staphylococcus equorum* (3), *Staphylococcus succinus* (1) and *Staphylococcus vitulinus* (1) (**Table S1**).

Among the 15 strains from the Gram-negative group, one belongs to Betaproteobacteria and 14 to Gammaproteobacteria class. The Betaproteobacteria isolated in this study (FME57), possess high level of identity with the 16S rRNA of *Advenella kashmirensis*, *A. incenata* and *A. mimigardefordensis* type strains (respectively 98.9, 99.25 and 99.48%) as presented **Figure C**.

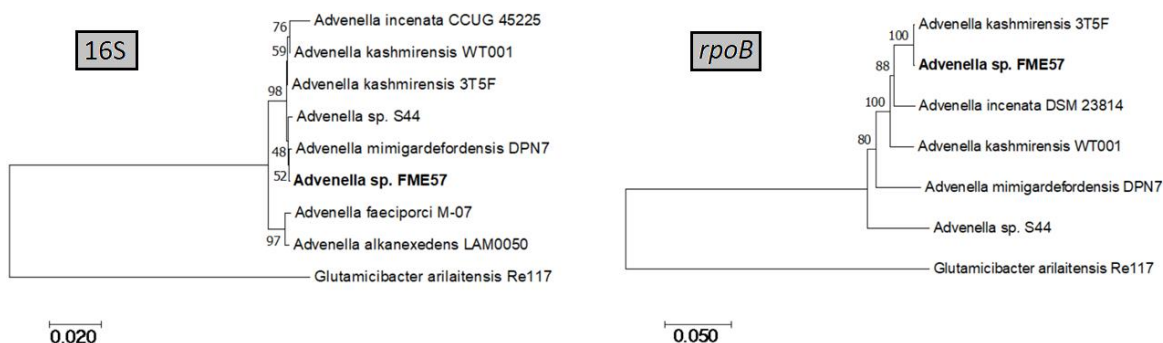


Figure C. Phylogenetic tree of *Advenella* genus based on 16S rRNA and *rpoB* gene alignments, including FME57 strain (isolated in this study) and its closest relatives.

Remarkably, the 16S rRNA of FME57 strain is 99.92% identical to that of *Advenella kashmirensis* 3T5F and 99.74% from *Advenella sp.* S44, which were isolated from cheese. Furthermore, its *rpoB* gene sequence best hit at NCBI was with *Advenella incenata* DSM 23814^T (96.5%) (**Table S1**), and it was only 96.26% with the *Advenella kashmirensis* WT001^T, while still is 100% identical with that of *Advenella kashmirensis* 3T5F. These data indicated that 3T5F and FME57 strains belongs to the same species, but may not be assigned reliably to *A. kashmirensis* (**Figure C**). Concerning isolates belonging to the Gammaproteobacteria group, they were distributed in seven genera: *Hafnia* (1), *Halomonas* (3), *Proteus* (1), *Pseudoalteromonas* (2), *Psychrobacter* (4) and *Vibrio* (1). Among these strains, three could be assigned unambiguously at the level of species considering the two marker genes data to yield *Hafnia alvei* (1), *Pseudomonas lundensis* (1) and *Vibrio casei* (1) (**Table 2**). In the case of *Halomonas* FME20 strain, its 16S rRNA and *rpoB* gene sequences display high homology, respectively 99.5 and 98.5%, with those of *Halomonas zhanjiangensis* DSM 21076^T (**Table 2**). FME20 may thus belong to this species or be closely related to, and this hypothesis deserve further analysis to be confirmed. The two last *Halomonas* strains, FME1 and FME16, remain unassigned to the species level since their 16S rRNA and *rpoB* genes display

levels of identity below the thresholds to all the defined species; however, they are closely related to JB37 and JB380 strains, respectively, which were isolated from cheese (Table S1, Fig. D).

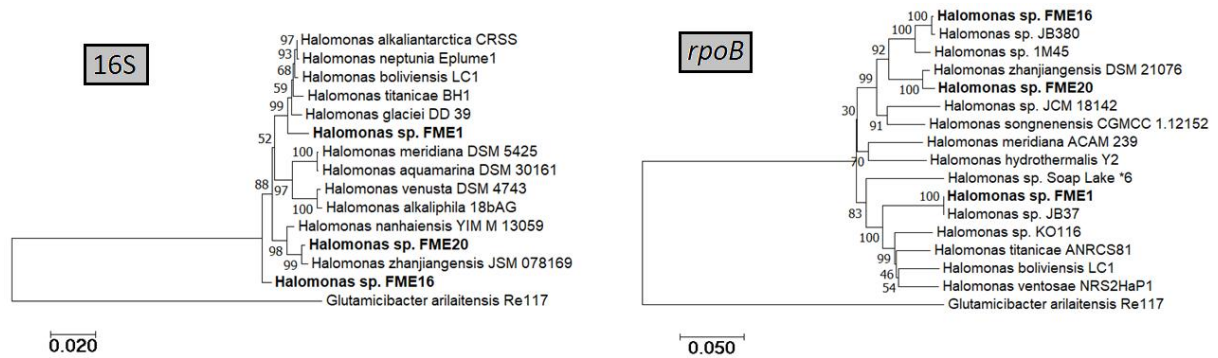


Figure D. Phylogenetic tree of *Halomonas* genus based on 16S rRNA and *rpoB* gene alignments, including FME1, FME16 and FME20 strains (isolated in this study) and their closest relatives.

Beside this, the *Proteus* isolate FME41 also remained unassigned because, although its 16S rRNA shares around 99% identity with the one of type strain, *Proteus hauseri* DSM 21076^T, the *rpoB* nucleotide sequences of these two strains share only 93.92% (Table 2, Figure E).

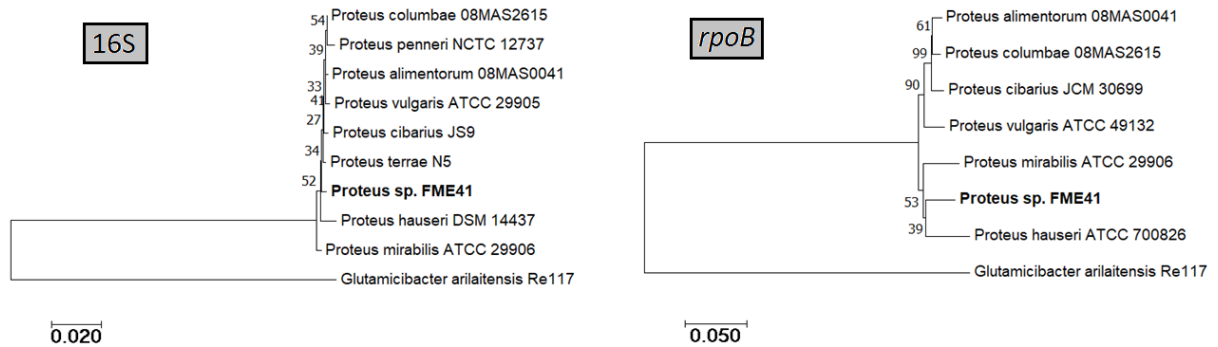


Figure E. Phylogenetic tree of *Proteus* genus based on 16S rRNA and *rpoB* gene alignments, including FME41 strain (isolated in this study) and its closest relatives.

In the same way, *Pseudomonas* FME51 strain could not be assigned reliably, although its 16S rRNA gene sequence shares around 99% identity with the one of *Pseudomonas litoralis* 2SM5^T, since its *rpoB* nucleotide sequence shares only 93.27% with this type strain (Table S1, Figure F).

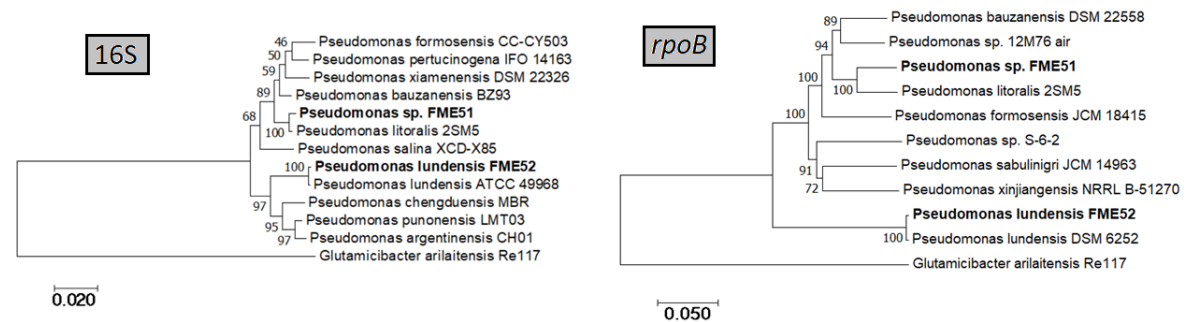


Figure F. Phylogenetic tree of *Pseudomonas* genus based on 16S rRNA and *rpoB* gene alignments, including FME51 and FME52 strains (isolated in this study) and their closest relatives.

Furthermore, *Pseudoalteromonas* FME14 and FME53 strains could not be reliably attributed to one species because the two marker genes share high identity (over the threshold) with different species and consequently their result are not congruent (**Table S1, Figure G**).

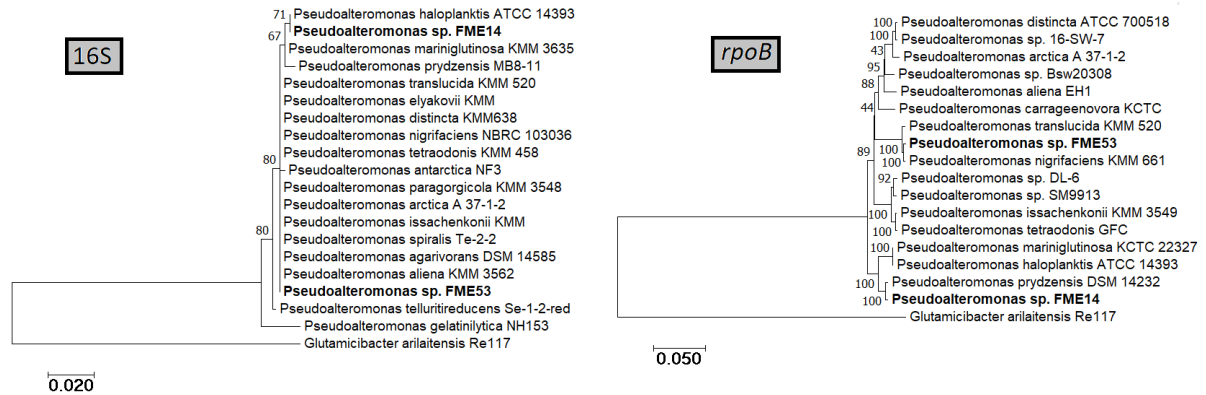


Figure G. Phylogenetic tree of *Pseudoalteromonas* genus based on 16S rRNA and *rpoB* gene alignments, including FME14 and FME53 strains (isolated in this study) and their closest relatives.

Finally, the four *Psychrobacter* strains remain unassigned because their marker gene sequences display insufficient levels of homology and/or non congruent results to delineate them as defined species (**Table S1, Figure H**).

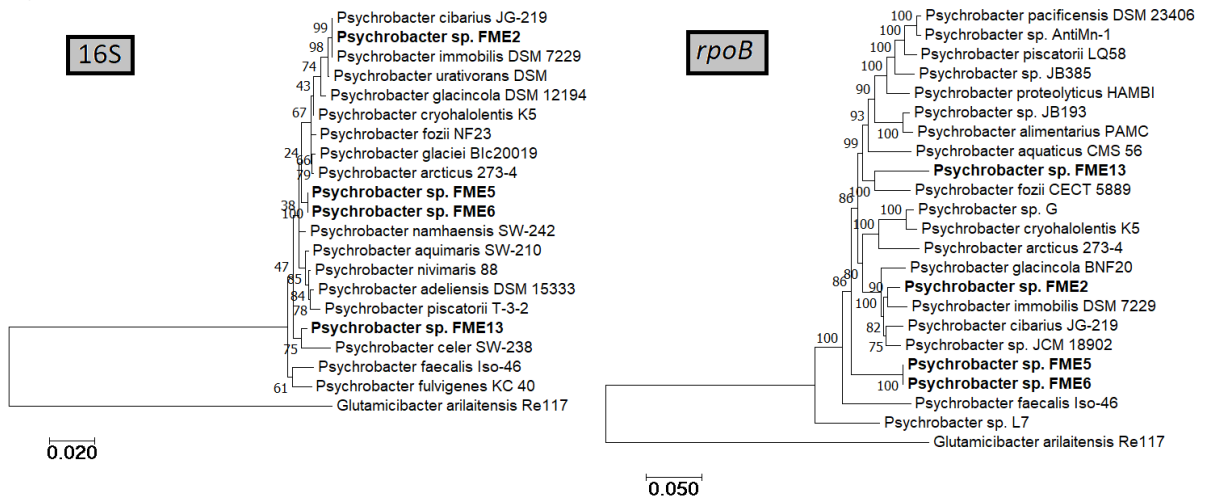


Figure H. Phylogenetic tree of *Psychrobacter* genus based on 16S rRNA and *rpoB* gene alignments, including FME2, FME5, FME6 and FME13 strains (isolated in this study) and their closest relatives.

Additional File 2. This supplementary information documents the main bacterial species of halophilic and halotolerant bacteria in food and their potential roles.

	Phylum	Genus	Food sources	Main species	Potential role or contribution to food	Authors
Gram-positive	Actinobacteria	<i>Glutamicibacter</i>	Cheese, milk	<i>G. arilaitensis</i> , <i>G. bergerei</i>	Contribution to the colour, flavour and texture properties in cheese or contaminant/ spoilage.	[1-3]
	Actinobacteria	<i>Brevibacterium</i>	Cheese, milk, salted food	<i>B. aurantiacum</i> , <i>B. antiquum</i> , <i>B. linens</i> , <i>B. casei</i> , <i>B. spp.</i>	Used as adjunct in cheese; proteolytic, lipolytic and esterase activities; production of antimicrobial substances, pigments and aromatic compounds.	[1, 4-10]
	Actinobacteria	<i>Corynebacterium</i>	Cheese, milk	<i>C. casei</i> , <i>C. variabile</i>	Contribution to the aroma and colour of surface-ripened cheeses.	[11-15]
	Actinobacteria	<i>Brachybacterium</i>	Fermented food, especially in cheese	<i>B. tyrofermentans</i> , <i>B. alimentarium</i>	Contribution to the flavour and colour in cheeses.	[16-18]
	Firmicutes	<i>Staphylococcus</i> (coagulase-negative)	Fermented foods, sausages and meat products	<i>S. equorum</i> , <i>S. succinus</i> , <i>S. xylosus</i>	Used as adjunct in cheese to improve its texture and bring particular flavors, rind coloration, proteolytic and lipolytic activities.	[5, 13, 19-22]
	Firmicutes	<i>Oceanobacillus</i>	Korean fermented food	<i>O. kimchii</i> , <i>O. gochujangensis</i>	Unknown function in food, likely derived from solar salts.	[23-25]
	Firmicutes	<i>Carnobacterium</i>	Cheese, milk, meat, fish, shrimp	<i>C. maltaromaticum</i> , <i>C. mobile</i> , <i>C. divergens</i>	Production of antimicrobial peptides and bacteriocins and production of volatile compounds that contribute for aroma or spoilage in food.	[26-29]
	Firmicutes	<i>Marinilactibacillus</i>	Cheese rinds, salted food, cheese brine	<i>M. psychrotolerans</i>	Unknown function in cheese, associated to spoilage in dry-cured hams.	[30-33]
Gram-negative	Proteobacteria	<i>Advenella</i>	Cheese, milk	<i>A. incenata</i> , <i>A. kashmirensis</i> , <i>A. spp.</i>	Potential to contribute positively to cheese ripening.	[34-36]
	Proteobacteria	<i>Pseudoalteromonas</i>	Cheese, Korean fermented food	<i>P. haloplanktis</i> , <i>P. spp</i>	Suggestion of play key roles in cheese rind microbial communities.	[37-40]
	Proteobacteria	<i>Hafnia</i>	Cheese, milk	<i>H. alvei</i>	Used as adjunct culture in cheese to improve cheese flavor.	[29, 39, 41, 42]
	Proteobacteria	<i>Proteus</i>	Cheese, milk	<i>P. vulgaris</i> , <i>P. mirabilis</i> , <i>P. hauseri</i> , <i>P. spp</i>	Technological interest as contribution to the organoleptic properties of cheese, but also spoiler or potential pathogen.	[43-47]
	Proteobacteria	<i>Halomonas</i>	Cheese, milk, meat products	<i>H. alkaliphila</i> , <i>H. variabilis</i> , <i>H. venusta</i> , <i>H. spp.</i>	Suggestions of positive impact during cheese ripening or spoilage bacteria.	[14, 34, 37, 48-50]
	Proteobacteria	<i>Psychrobacter</i>	Cheese, sea food, meat products	<i>P. celer</i> , <i>P. cibarius</i> , <i>P. spp.</i>	Production of volatile compounds could have a positive impact during the cheese ripening or negative impact as in the food spoilage.	[5, 47, 50-52]
	Proteobacteria	<i>Pseudomonas</i>	Meat, poultry, milk and dairy products	<i>P. aeruginosa</i> , <i>P. lundensis</i> , <i>P. fluorescens</i> , <i>P. spp.</i>	Strongly proteolytic activity that cause undesirable odor and flavors, frequently associated as contaminant or spoilage.	[53-57]
	Proteobacteria	<i>Vibrio</i>	Cheese, seafood	<i>V. casei</i> , <i>V. litoralis</i> , <i>V. spp.</i>	Suggestion of play a role in the cheese ripening process.	[14, 30, 58-60]

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Table S1. Potential assignment of 35 representative isolates using 16S rRNA and *rpoB* genes.

	Isolate in this study	Phylum	Class	Order	Best score blast of 16S rRNA [Ref RNA Sequences]			Best score blast of <i>rpoB</i> [Nucleotide]		
					Strain	Query cover (%)	Identity (%)	Strain	Query cover (%)	Identity (%)
Gram-positive strains	<i>Brachy bacterium</i> sp. FME24	Actinobacteria	Actinobacteria	Micrococcales	<i>Brachy bacterium</i> tyrofermentans CNRZ 926 ^T	99	99.07	<i>Brachy bacterium</i> tyrofermentans CNRZ 926 ^T	100	93.43
	<i>Brachy bacterium</i> tyrofermentans FME25	Actinobacteria	Actinobacteria	Micrococcales	<i>Brachy bacterium</i> tyrofermentans CNRZ 926 ^T	99	99.6	<i>Brachy bacterium</i> tyrofermentans CNRZ 926 ^T	100	100
	<i>Brevibacterium aurantiacum</i> FME34	Actinobacteria	Actinobacteria	Actinomycetales	<i>Brevibacterium aurantiacum</i> NCDO 739 ^T	95	98.58	<i>Brevibacterium aurantiacum</i> ATCC 9175 ^T	100	100
	<i>Brevibacterium aurantiacum</i> FME43	Actinobacteria	Actinobacteria	Actinomycetales	<i>Brevibacterium aurantiacum</i> NCDO 739 ^T	95	98.44	<i>Brevibacterium aurantiacum</i> ATCC 9175 ^T	100	99.83
	<i>Brevibacterium aurantiacum</i> FME45	Actinobacteria	Actinobacteria	Actinomycetales	<i>Brevibacterium aurantiacum</i> NCDO 739 ^T	95	100	<i>Brevibacterium aurantiacum</i> ATCC 9175 ^T	100	100
	<i>Brevibacterium aurantiacum</i> FME48	Actinobacteria	Actinobacteria	Actinomycetales	<i>Brevibacterium aurantiacum</i> NCDO 739 ^T	95	98.85	<i>Brevibacterium aurantiacum</i> ATCC 9175 ^T	100	99.71
	<i>Brevibacterium aurantiacum</i> FME49	Actinobacteria	Actinobacteria	Actinomycetales	<i>Brevibacterium aurantiacum</i> NCDO 739 ^T	96	98.19	<i>Brevibacterium aurantiacum</i> ATCC 9175 ^T	100	99.63
	<i>Brevibacterium aurantiacum</i> FME9	Actinobacteria	Actinobacteria	Actinomycetales	<i>Brevibacterium aurantiacum</i> NCDO 739 ^T	95	98.58	<i>Brevibacterium aurantiacum</i> ATCC 9175 ^T	100	99.97
	<i>Brevibacterium</i> sp. FME17	Actinobacteria	Actinobacteria	Actinomycetales	<i>Brevibacterium antiquum</i> KM Ac-2118 ^T	96	98.46	<i>Brevibacterium antiquum</i> KM Ac-2118 ^T	100	95.23
	<i>Brevibacterium</i> sp. FME37	Actinobacteria	Actinobacteria	Actinomycetales	<i>Brevibacterium antiquum</i> KM Ac-2118 ^T	96	98.26	<i>Brevibacterium antiquum</i> KM Ac-2118 ^T	100	95.26
	<i>Corynebacterium casei</i> FME59	Actinobacteria	Actinobacteria	Corynebacteriales	<i>Corynebacterium casei</i> LMG S-19264 ^T	99	99.8	<i>Corynebacterium casei</i> LMG S-19264 ^T	100	99.49
	<i>Glutamicibacter arilaitensis</i> FME22	Actinobacteria	Actinobacteria	Actinomycetales	<i>Glutamicibacter arilaitensis</i> Re117 ^T	99	100	<i>Glutamicibacter arilaitensis</i> Re117 ^T	100	99.89
	<i>Carnobacterium mobile</i> FME4	Firmicutes	Bacilli	Lactobacillales	<i>Carnobacterium mobile</i> DSM 4848 ^T	94	99.8	<i>Carnobacterium mobile</i> DSM 4848 ^T	100	99.78
	<i>Marinilactibacillus psychrotolerans</i> FME56	Firmicutes	Bacilli	Lactobacillales	<i>Marinilactibacillus psychrotolerans</i> NBRC 100002 ^T	94	99.93	<i>Marinilactibacillus psychrotolerans</i> NBRC 100002 ^T	100	98.33
	<i>Oceanobacillus oncorhynchi</i> FME55	Firmicutes	Bacilli	Bacillales	<i>Oceanobacillus oncorhynchi</i> subsp. <i>incaldanensis</i> ^T	95	99.6	<i>Oceanobacillus oncorhynchi</i> Oc5	100	99.83
	<i>Staphylococcus equorum</i> FME18	Firmicutes	Bacilli	Bacillales	<i>Staphylococcus equorum</i> PA 231 ^T	95	100	<i>Staphylococcus equorum</i> NCTC 12414 ^T	100	99.92
	<i>Staphylococcus equorum</i> FME19	Firmicutes	Bacilli	Bacillales	<i>Staphylococcus equorum</i> PA 231 ^T	95	100	<i>Staphylococcus equorum</i> NCTC 12414 ^T	100	98.76
<i>Staphylococcus equorum</i> FME58	Firmicutes	Bacilli	Bacillales	<i>Staphylococcus equorum</i> PA 231 ^T	95	100	<i>Staphylococcus equorum</i> NCTC 12414 ^T	100	99.63	
<i>Staphylococcus vitulinus</i> FME39	Firmicutes	Bacilli	Bacillales	<i>Staphylococcus vitulinus</i> ATCC 51145 ^T	95	99.93	<i>Staphylococcus vitulinus</i> DSM 15615 ^T	100	99.77	
<i>Staphylococcus succinus</i> FME10	Firmicutes	Bacilli	Bacillales	<i>Staphylococcus succinus</i> subsp. <i>succinus</i> AMG-D1 ^T	98	99.81	<i>Staphylococcus succinus</i> subsp. <i>succinus</i> DSM 14617 ^T	100	99.5	
Gram-negative strains	<i>Advenella</i> sp. FME57	Proteobacteria	Betaproteobacteria	Burkholderiales	<i>Advenella kashmirensis</i> WT001 ^T	99	98.9	<i>Advenella incenata</i> DSM 23814 ^T	100	96.48
	<i>Hafnia alvei</i> FME31	Proteobacteria	Gammaproteobacteria	Enterobacteriales	<i>Hafnia alvei</i> ATCC 13337 ^T	95	99.66	<i>Hafnia alvei</i> ATCC 13337 ^T	100	100
	<i>Halomonas</i> sp. FME1	Proteobacteria	Gammaproteobacteria	Oceanospirillales	<i>Halomonas alkaliantarctica</i> CRSS ^T	98	98.75	<i>Halomonas</i> sp. JB37	100	100
	<i>Halomonas</i> sp. FME16	Proteobacteria	Gammaproteobacteria	Oceanospirillales	<i>Halomonas alkaliantarctica</i> CRSS ^T	98	98.09	<i>Halomonas</i> sp. JB380	100	99.63
	<i>Halomonas</i> sp. FME20	Proteobacteria	Gammaproteobacteria	Oceanospirillales	<i>Halomonas chanjiangensis</i> DSM 21076 ^T	93	99.52	<i>Halomonas chanjiangensis</i> DSM 21076 ^T	100	98.55
	<i>Proteus</i> sp. FME41	Proteobacteria	Gammaproteobacteria	Enterobacteriales	<i>Proteus hauseri</i> DSM 14437 ^T	97	99.07	<i>Proteus hauseri</i> ATCC 700826 ^T	100	93.92
	<i>Pseudoalteromonas</i> sp. FME14	Proteobacteria	Gammaproteobacteria	Alteromonadales	<i>Pseudoalteromonas mariniglutinosa</i> KMM 3635 ^T	99	99.44	<i>Pseudoalteromonas prydzensis</i> DSM 14232 ^T	100	99.33
	<i>Pseudoalteromonas</i> sp. FME53	Proteobacteria	Gammaproteobacteria	Alteromonadales	<i>Pseudoalteromonas distincta</i> KMM 638 ^T	97	99.73	<i>Pseudoalteromonas nigrifaciens</i> KMM 661 ^T	100	99.73
	<i>Pseudomonas lundensis</i> FME52	Proteobacteria	Gammaproteobacteria	Pseudomonadales	<i>Pseudomonas lundensis</i> ATCC 49968 ^T	97	99.21	<i>Pseudomonas lundensis</i> W22b	100	99.85
	<i>Pseudomonas</i> sp. FME51	Proteobacteria	Gammaproteobacteria	Pseudomonadales	<i>Pseudomonas litoralis</i> 2SM5 ^T	93	99.19	<i>Pseudomonas litoralis</i> 2SM5 ^T	100	93.27
	<i>Psychrobacter</i> sp. FME13	Proteobacteria	Gammaproteobacteria	Pseudomonadales	<i>Psychrobacter arcticus</i> 273-4 ^T	99	98.18	<i>Psychrobacter</i> sp. 4Dc	99	91.14
	<i>Psychrobacter</i> sp. FME2	Proteobacteria	Gammaproteobacteria	Pseudomonadales	<i>Psychrobacter cibarius</i> JG-219 ^T	96	100	<i>Psychrobacter cibarius</i> W1	100	96.38
	<i>Psychrobacter</i> sp. FME5	Proteobacteria	Gammaproteobacteria	Pseudomonadales	<i>Psychrobacter arcticus</i> 273-4 ^T	99	98.96	<i>Psychrobacter proteolyticus</i> HAMB1_2948	99	90.34
	<i>Psychrobacter</i> sp. FME6	Proteobacteria	Gammaproteobacteria	Pseudomonadales	<i>Psychrobacter arcticus</i> 273-4 ^T	99	99.03	<i>Psychrobacter proteolyticus</i> HAMB1_2948	99	90.34
	<i>Vibrio casei</i> FME29	Proteobacteria	Gammaproteobacteria	Vibrionales	<i>Vibrio casei</i> DSM 22364 ^T	99	98.91	<i>Vibrio casei</i> DSM 22364 ^T	100	100

Table S2. General features of the 35 genomes sequenced in this study.

	Species	Strain	No of contigs	Size (base pairs)	GC content (%)	N50 ^a	L50 ^b	Number of subsystems	Number of coding sequences	Number of RNAs	Accession
Gram-positive strains	<i>Brachy bacterium sp.</i>	FME24	67	4811703	69.3	240808	8	296	4540	51	JABUXV000000000
	<i>Brachy bacterium tyrofermentans</i>	FME25	108	4609434	70.0	165603	12	285	4364	56	JABUXW000000000
	<i>Brevibacterium aurantiacum</i>	FME34	65	4124821	62.8	199214	7	302	3869	52	JABUXX000000000
	<i>Brevibacterium aurantiacum</i>	FME43	85	4167564	62.8	169753	8	306	3925	52	JABUXY000000000
	<i>Brevibacterium aurantiacum</i>	FME45	115	4191006	62.8	153278	8	304	3987	53	JABUXZ000000000
	<i>Brevibacterium aurantiacum</i>	FME48	75	3975013	62.9	192326	7	305	3698	53	JABUYA000000000
	<i>Brevibacterium aurantiacum</i>	FME49	92	4096764	62.8	198846	7	307	3833	53	JABUYB000000000
	<i>Brevibacterium aurantiacum</i>	FME9	60	4084029	62.7	194502	7	305	3811	53	JABUYC000000000
	<i>Brevibacterium sp.</i>	FME17	87	4439845	62.2	160515	9	305	4256	52	JABUYD000000000
	<i>Brevibacterium sp.</i>	FME37	65	4321034	62.1	216643	7	312	4133	54	JABUYE000000000
	<i>Corynebacterium casei</i>	FME59	90	3041289	55.4	106800	8	261	2926	54	JABUYG000000000
	<i>Glutamicibacter arilaitensis</i>	FME22	183	3760962	59.3	63743	18	300	3784	64	JABUYH000000000
	<i>Carnobacterium mobile</i>	FME4	98	2542362	36.7	53528	14	231	2471	43	JABUYF000000000
	<i>Marinilactibacillus psychrotolerans</i>	FME56	473	2534696	35.5	30687	26	228	2555	48	JABUYJ000000000
	<i>Oceanobacillus orcorhynchi</i>	FME55	76	4914074	38.8	153756	10	362	5127	66	JABUYK000000000
	<i>Staphylococcus equorum</i>	FME18	25	2676219	32.9	461095	3	288	2588	51	JABUYO00000000
	<i>Staphylococcus equorum</i>	FME19	57	2849813	33.0	410660	3	292	2774	51	JABUYP000000000
<i>Staphylococcus equorum</i>	FME58	33	2776149	32.9	308559	4	289	2738	34	JABUYQ000000000	
<i>Staphylococcus vitulinus</i>	FME39	141	2655894	32.7	38983	15	293	2715	44	JABUYR000000000	
<i>Staphylococcus succinus</i>	FME10	18	2722055	32.8	554036	2	296	2585	51	JABUYS000000000	
Gram-negative strains	<i>Advenella sp.</i>	FME57	59	4738585	54.7	221123	8	345	4886	41	JABUXU000000000
	<i>Hafnia alvei</i>	FME31	99	4876576	48.9	188191	8	401	4749	71	JABUYI000000000
	<i>Halomonas sp.</i>	FME1	57	3858166	53.8	227665	7	352	3696	45	RRZD000000000
	<i>Halomonas sp.</i>	FME16	62	3710911	53.9	157431	7	364	3606	59	RRZC000000000
	<i>Halomonas sp.</i>	FME20	194	4221656	54.2	67999	20	366	4065	63	RRZB000000000
	<i>Proteus sp.</i>	FME41	83	4176124	38.0	182411	8	366	3863	77	JABUYL000000000
	<i>Pseudoalteromonas prydzensis</i>	FME14	243	4899689	41.0	56441	29	358	4635	61	RRZA000000000
	<i>Pseudoalteromonas nigrifaciens</i>	FME53	232	4279332	39.8	55370	27	356	4092	62	RRYZ000000000
	<i>Pseudomonas lundensis</i>	FME52	230	5279217	58.2	60527	28	383	5193	59	JABUYM000000000
	<i>Pseudomonas sp.</i>	FME51	57	4786847	54.2	360315	5	374	4621	63	JABUYN000000000
	<i>Psychrobacter sp.</i>	FME13	419	3708697	41.8	20335	50	286	3557	47	RRYV000000000
	<i>Psychrobacter sp.</i>	FME2	59	3367099	42.7	154167	7	287	3007	47	RRYY000000000
	<i>Psychrobacter sp.</i>	FME5	295	3373895	42.8	24719	38	284	3117	45	RRYX000000000
	<i>Psychrobacter sp.</i>	FME6	268	3361643	42.8	30966	35	277	3067	45	RRYW000000000
	<i>Vibrio casei</i>	FME29	115	3833673	40.5	106021	11	352	3631	64	JABUYT000000000

^aN50 is the length of contigs that collectively cover at least 50% of the assembly.

^bL50 is the number of contigs equal to or longer than N50.

Table S3. Average Nucleotide Identity (ANI) with close relatives of strains that marker genes (16S rRNA and *rpoB*) were unable to reliably assign to a described species. ANI values greater than or equal to 95% are shown in green.

	<i>Brachybacterium</i> <i>sp.</i> FME24	<i>Brachybacterium</i> <i>sp.</i> FME25	<i>Brachybacterium</i> <i>tyrofermentans</i> CNRZ926 ^T	<i>Brachybacterium</i> <i>alimentarium</i> 908_11
<i>Brachybacterium sp.</i> FME24		82.38	83.17	81.28
<i>Brachybacterium sp.</i> FME25			95.89	82.78
<i>Brachybacterium tyrofermentans</i> CNRZ926 ^T				84.99
<i>Brachybacterium alimentarium</i> 908_11				

	<i>Brevibacterium</i> <i>sp.</i> FME17	<i>Brevibacterium</i> <i>sp.</i> FME37	<i>Brevibacterium</i> <i>sp.</i> 239c	<i>Brevibacterium</i> <i>antiquum</i> CNRZ 918	<i>Brevibacterium</i> <i>sandarakinum</i> DSM 22082 ^T	<i>Brevibacterium</i> <i>aurantiacum</i> ATCC 9175 ^T
<i>Brevibacterium sp.</i> FME17		97.93	97.09	85.47	84.58	85.7
<i>Brevibacterium sp.</i> FME37			97.38	85.76	84.65	86.18
<i>Brevibacterium sp.</i> 239c				85.53	84.52	85.33
<i>Brevibacterium antiquum</i> CNRZ 918					85.42	86.72
<i>Brevibacterium sandarakinum</i> DSM 22082 ^T						85.21
<i>Brevibacterium aurantiacum</i> ATCC 9175 ^T						

	<i>Advenella sp.</i> FME57	<i>Advenella</i> <i>kashmirensis</i> 3T5F	<i>Advenella incenata</i> DSM 23814 ^T	<i>Advenella</i> <i>kashmirensis</i> WT001 ^T	<i>Advenella</i> <i>mimigardefordensis</i> DPN7 ^T	<i>Advenella sp.</i> S44
<i>Advenella sp.</i> FME57		98.57	90.42	88.68	84.4	83.92
<i>Advenella kashmirensis</i> 3T5F			90.57	89	84.19	83.81
<i>Advenella incenata</i> DSM 23814 ^T				88.89	83.85	82.77
<i>Advenella kashmirensis</i> WT001 ^T					83.81	82.5
<i>Advenella mimigardefordensis</i> DPN7 ^T						80.47
<i>Advenella sp.</i> S44						

	<i>Halomonas</i> <i>sp.</i> FME16	<i>Halomonas</i> <i>sp.</i> JB380	<i>Halomonas</i> <i>sp.</i> 1M45	<i>Halomonas</i> <i>sp.</i> FME20	<i>Halomonas zhanjiangensis</i> DSM 21076 ^T	<i>Halomonas songnenensis</i> CGMCC 1.12152	<i>Halomonas sp.</i> JCM 18142
<i>Halomonas sp.</i> FME16		97.46	95.89	82.24	82.23	78.6	80.44
<i>Halomonas sp.</i> JB380			95.01	81.93	82.18	78.59	80.11
<i>Halomonas sp.</i> 1M45				82.61	82.52	78.68	80.47
<i>Halomonas sp.</i> FME20					93.22	79.48	81.62
<i>Halomonas zhanjiangensis</i> DSM 21076 ^T						79.76	81.98
<i>Halomonas songnenensis</i> CGMCC 1.12152							80.97
<i>Halomonas sp.</i> JCM 18142							

	<i>Halomonas sp.</i> FME1	<i>Halomonas sp.</i> JB37	<i>Halomonas sp.</i> KO116	<i>Halomonas</i> <i>titanicae</i> BH1 ^T	<i>Halomonas</i> <i>boliviensis</i> LC1 ^T	<i>Halomonas ventosae</i> NRS2HaP1
<i>Halomonas sp.</i> FME1		99.27	79.64	79.69	79.97	79.78
<i>Halomonas sp.</i> JB37			79.73	79.78	79.98	79.84
<i>Halomonas sp.</i> KO116				85.33	84.17	85.91
<i>Halomonas titanicae</i> BH1 ^T					86.63	87.76
<i>Halomonas boliviensis</i> LC1 ^T						86.93
<i>Halomonas ventosae</i> NRS2HaP1						

	<i>Proteus sp.</i> FME41	<i>Proteus vulgaris</i> ATCC 49132	<i>Proteus cibarius</i> JCM 30699 ^T	<i>Proteus hauseri</i> ATCC 700826 ^T	<i>Proteus mirabilis</i> ATCC 29906 ^T
<i>Proteus sp.</i> FME41		88.28	88.62	82.91	82.26
<i>Proteus vulgaris</i> ATCC 49132			89.42	82.75	82.12
<i>Proteus cibarius</i> JCM 30699				82.94	82.36
<i>Proteus hauseri</i> ATCC 700826 ^T					81.3
<i>Proteus mirabilis</i> ATCC 29906 ^T					

	<i>Pseudomonas</i> <i>sp.</i> FME51	<i>Pseudomonas</i> <i>litoralis</i> 2SM5 ^T	<i>Pseudomonas</i> <i>bauzanensis</i> DSM 22558 ^T	<i>Pseudomonas</i> <i>saudimassiliensis</i> 12M76 air ^T	<i>Pseudomonas</i> <i>sp.</i> MYb185	<i>Pseudomonas</i> <i>formosensis</i> JCM 18415 ^T
<i>Pseudomonas sp.</i> FME51		87.70	82.28	80.67	80.54	77.67
<i>Pseudomonas litoralis</i> 2SM5 ^T			82.37	81.24	80.87	78.08
<i>Pseudomonas bauzanensis</i> DSM 22558 ^T				83.42	81.88	79.55
<i>Pseudomonas saudimassiliensis</i> 12M76 air ^T					81.29	79.58
<i>Pseudomonas sp.</i> MYb185						80.12
<i>Pseudomonas formosensis</i> JCM 18415 ^T						

	<i>Pe.</i> <i>nigrifaciens</i> FME53	<i>Pe.</i> <i>nigrifaciens</i> NCTC 10691 ^T	<i>Pe.</i> <i>translucida</i> KMM 520 ^T	<i>Pe.</i> <i>arctica</i> A37-1-2 ^T	<i>Pe.</i> <i>distincta</i> ATCC 700518 ^T	<i>Pe.</i> <i>prydzensis</i> FME14	<i>Pe.</i> <i>prydzensis</i> DSM 14232 ^T	<i>Pe.</i> <i>elyakovii</i> ATCC 700519 ^T	<i>Pe.</i> <i>mariniglutinosa</i> KCTC 22327	<i>Pe.</i> <i>haloplanktis</i> ATCC 14393 ^T
<i>Pe. nigrifaciens</i> FME53		98.31	96.12	81.21	80.81	76.71	76.09	70.28	75.58	75.72
<i>Pe. nigrifaciens</i> NCTC 10691 ^T			95.86	81.28	80.64	76.58	76	70.6	75.72	75.83
<i>Pe. translucida</i> KMM 520 ^T				80.75	80.62	76.07	75.76	70.59	75.56	75.85
<i>Pe. arctica</i> A37-1-2 ^T					93.31	76.22	75.8	70.51	75.44	75.43
<i>Pe. distincta</i> ATCC 700518 ^T						75.78	75.45	70.44	75.2	75.17
<i>Pe. prydzensis</i> FME14							95.97	70.38	85.31	85.27
<i>Pe. prydzensis</i> DSM 14232 ^T								70.2	85.49	85.42
<i>Pe. elyakovii</i> ATCC 700519 ^T									70.07	70.15
<i>Pe. mariniglutinosa</i> KCTC 22327										99.98
<i>Pe. haloplanktis</i> ATCC 14393 ^T										

	<i>Psychrobacter</i> sp. FME2	<i>Psychrobacter</i> <i>immobilis</i> DSM 7229 ^T	<i>Psychrobacter</i> <i>cibarius</i> JG- 219 ^T	<i>Psychrobacter</i> <i>glacincola</i> BNF20	<i>Psychrobacter</i> sp. JCM 18902	<i>Psychrobacter</i> sp. P11F6
<i>Psychrobacter</i> sp. FME2		95.40	95.58	95.42	95.23	95.49
<i>Psychrobacter immobilis</i> DSM 7229 ^T			95.45	95.66	95.77	95.66
<i>Psychrobacter cibarius</i> JG-219				95.67	95.52	95.59
<i>Psychrobacter glacincola</i> BNF20					95.53	95.64
<i>Psychrobacter</i> sp. JCM 18902						95.63
<i>Psychrobacter</i> sp. P11F6						

	<i>Psychrobacter</i> sp. FME5	<i>Psychrobacter</i> sp. FME6	<i>Psychrobacter</i> sp. FME13	<i>Psychrobacter</i> <i>faecalis</i> Iso-46 ^T	<i>Psychrobacter</i> <i>fozii</i> CECT 5889	<i>Psychrobacter</i> <i>aquaticus</i> CMS 56 ^T	<i>Psychrobacter pacificensis</i> DSM 23406 ^T
<i>Psychrobacter</i> sp. FME5		98.28	80.87	80.67	79.48	79.85	78.88
<i>Psychrobacter</i> sp. FME6			80.56	80.50	79.38	79.71	78.72
<i>Psychrobacter</i> sp. FME13				79.42	82.67	79.28	79.26
<i>Psychrobacter faecalis</i> Iso-46 ^T					78.24	78.63	78.2
<i>Psychrobacter fozii</i> CECT 5889						79.82	79.09
<i>Psychrobacter aquaticus</i> CMS 56 ^T							79.21
<i>Psychrobacter pacificensis</i> DSM 23406 ^T							

Table S4. Metadata of 25 *Brevibacterium aurantiacum* and 43 *Staphylococcus equorum* used for pan-genome analyses.

Species	Strain	Graph Reference	Source	Country	Bioproject	BioSample	GenBank accession number	No of contigs	Size (bp)	Min contig size (bp)	Authors
<i>B. aurantiacum</i>	FME9	Ba_FME9	Cheese rind	France	PRJNA501839	SAMN15150938	JABUYC000000000	60	4084029	327	This study
<i>B. aurantiacum</i>	FME34	Ba_FME34	Cheese rind	France	PRJNA501839	SAMN15150933	JABUXX000000000	65	4124821	301	This study
<i>B. aurantiacum</i>	FME43	Ba_FME43	Cheese rind	France	PRJNA501839	SAMN15150934	JABUXY000000000	85	4167564	302	This study
<i>B. aurantiacum</i>	FME45	Ba_FME45	Cheese rind	France	PRJNA501839	SAMN15150935	JABUXZ000000000	115	4191006	307	This study
<i>B. aurantiacum</i>	FME48	Ba_FME48	Cheese rind	France	PRJNA501839	SAMN15150936	JABUYA000000000	75	3975013	315	This study
<i>B. aurantiacum</i>	FME49	Ba_FME49	Cheese rind	France	PRJNA501839	SAMN15150937	JABUYB000000000	92	4096764	347	This study
<i>B. aurantiacum</i>	6 (3)	Ba_6_3	Cheese	France	PRJNA224116	SAMEA103892641	FXYZ01000001	91	4162132	1028	[1]
<i>B. aurantiacum</i>	8 (6)	Ba_8_6	Cheese	France	PRJNA224116	SAMEA103892642	FXZI01000001	97	4455163	1002	[1]
<i>B. aurantiacum</i>	ATCC 9175	Ba_ATCC_9175	Cheese	France	PRJNA224116	SAMEA103737418	FXZB000000000	70	4147494	1029	[1]
<i>B. aurantiacum</i>	CNRZ 920	Ba_CNRZ_920	Cheese	France	PRJEB19800	SAMEA103586668	FXZG000000000	73	3991882	1049	[1]
<i>B. aurantiacum</i>	SMQ-1335	Ba_SMQ_1335	Industrial dairy strain	Canada	PRJNA340866	SAMN05714647	CP017150	1	4209935	4209935	de Melo <i>et al.</i> (direct submission)
<i>B. aurantiacum</i>	SMQ-1419	Ba_SMQ_1419	Cheese	Canada	PRJNA421979	SAMN08162086	CP025333	1	4038634	4038634	[2]
<i>B. aurantiacum</i>	SMQ-1420	Ba_SMQ_1420	Cheese	Canada	PRJNA421981	SAMN08162094	CP025334	1	4328723	4328723	[2]
<i>B. aurantiacum</i>	SMQ-1418	Ba_SMQ_1418	Cheese	Canada	PRJNA421978	SAMN08162084	CP025331	1	4193206	4193206	[2]
<i>B. aurantiacum</i>	SMQ-1417	Ba_SMQ_1417	Cheese	Canada	PRJNA421977	SAMN08162081	CP025330	1	4424341	4424341	[2]
<i>B. aurantiacum</i>	SMQ-1421	Ba_SMQ_1421	Cheese	Canada	PRJNA421980	SAMN08162087	CP025332	1	4085228	4085228	[2]
<i>B. aurantiacum</i>	341_13	Ba_341_13	Cheese rind	Spain	PRJNA387187	SAMN07141149	NRHB000000000	45	3875658	1080	[3]
<i>B. aurantiacum</i>	876_7	Ba_876_7	Cheese rind	France	PRJNA387187	SAMN07141152	QPGD000000000	52	4107072	1139	[3]
<i>B. aurantiacum</i>	JB5	Ba_JB5	Cheese rind	USA	PRJNA387187	SAMN07141170	NRGX000000000	1	4310614	4310614	[3]
<i>B. aurantiacum</i>	962_8	Ba_962_8	Cheese rind	USA	PRJNA387187	SAMN07141156	NRGQ000000000	68	4182269	1002	[3]
<i>B. aurantiacum</i>	947_7	Ba_947_7	Cheese rind	USA	PRJNA387187	SAMN07141155	NRGP000000000	58	4099087	1000	[3]
<i>B. aurantiacum</i>	908_7	Ba_908_7	Cheese rind	France	PRJNA387187	SAMN07141154	QPGE000000000	59	3994456	1052	[3]
<i>B. aurantiacum</i>	738_8	Ba_738_8	Cheese rind	USA	PRJNA387187	SAMN07141150	NRHA000000000	56	4128221	1034	[3]
<i>B. aurantiacum</i>	862_7	Ba_862_7	Cheese rind	USA	PRJNA387187	SAMN07141151	QPGC000000000	103	4058475	1002	[3]
<i>B. aurantiacum</i>	900_6	Ba_900_6	Cheese rind	France	PRJNA387187	SAMN07141153	NRGO000000000	58	4029828	1122	[3]

Species	Strain	Graph Reference	Source	Country	Bioproject	BioSample	GenBank accession number	No of contigs	Size (bp)	Min contig size (bp)	Authors
<i>S. equorum</i>	FME18	Se_FME18	Cheese rind	France	PRJNA501839	SAMN15150950	JABUYO000000000	25	2676219	308	This study
<i>S. equorum</i>	FME19	Se_FME19	Cheese rind	France	PRJNA501839	SAMN15150951	JABUYP000000000	57	2849813	302	This study
<i>S. equorum</i>	FME58	Se_FME58	Cheese rind	France	PRJNA501839	SAMN15150952	JABUYQ000000000	33	2776149	387	This study
<i>S. equorum equorum</i>	Mu2	Se_Mu2	Cheese rind	France	PRJEA88899	SAMEA2272469	CAJL01000030	62	2927855	223	[4]
<i>S. equorum</i>	908_10	Se_908_10	Cheese rind	France	PRJNA301343	SAMN04244248	LNPY000000000	30	2927171	658	[4]
<i>S. equorum</i>	876_5	Se_876_5	Cheese rind	France	PRJNA301343	SAMN07333888	NMOI000000000	54	2820997	236	[4]
<i>S. equorum</i>	900_4	Se_900_4	Cheese rind	France	PRJNA301343	SAMN04244257	LNDI000000000	73	2881045	230	[4]
<i>S. equorum</i>	862_5	Se_862_5	Cheese rind	USA	PRJNA301343	SAMN04244252	LNMY000000000	53	2957130	220	[4]
<i>S. equorum</i>	962_6	Se_962_6	Cheese rind	USA	PRJNA301343	SAMN04244251	LNQA000000000	87	2909961	208	[4]
<i>S. equorum</i>	947_12	Se_947_12	Cheese rind	USA	PRJNA301343	SAMN04244250	LNPZ000000000	52	2909320	203	[4]
<i>S. equorum</i>	738_7	Se_738_7	Cheese rind	USA	PRJNA301343	SAMN04244247	LNPX000000000	117	3070780	229	[4]
<i>S. equorum</i>	341_10	Se_341_10	Cheese rind	Spain	PRJNA301343	SAMN04244258	LNNN000000000	64	2765265	220	[4]
<i>S. equorum</i>	BC9	Se_BC9	Cheese rind	USA	PRJNA301343	SAMN04244255	LNNB000000000	46	2899774	221	[4]
<i>S. equorum linens</i>	DSM 15097	Se_DSM_15097	Cheese rind	Germany	PRJNA339206	SAMN05978022	PPQL000000000	101	2768268	269	[4]
<i>S. equorum</i>	OffWhite_SAM	Se_White_SAM	Cheese rind	France	PRJNA301343	SAMN04244254	LNNA000000000	47	2821632	517	[4]
<i>S. equorum</i>	White_SAM	Se_OffWhite_SAM	Cheese rind	France	PRJNA301343	SAMN04244253	LNMZ000000000	90	2847069	576	[4]
<i>S. equorum</i>	BC3	Se_BC3	Cheese rind	USA	PRJNA301343	SAMN04244256	LNNC000000000	84	2757703	506	[4]
<i>S. equorum</i>	UMC-CNS-924	Se_UMC_CNS924	Milk	USA	PRJNA211360	SAMN02471902	AVBD000000000	39	2700865	293	Calcutt <i>et al.</i> (direct submission)
<i>S. equorum</i>	KS1039	Se_KS1039	Salted fermented food	South Korea	PRJNA299343	SAMN04196999	CP013114	1	2822193	2822193	Jeong <i>et al.</i> (direct submission)
<i>S. equorum</i>	C2014	Se_C2014	Salted fermented food	South Korea	PRJNA306198	SAMN04363544	CP013714	6	2930519	7313	Jeong <i>et al.</i> (direct submission)
<i>S. equorum</i>	KM1031	Se_KM1031	Salted fermented food	South Korea	PRJNA306199	SAMN04364120	CP013980	4	2792203	2638	Jeong <i>et al.</i> (direct submission)
<i>S. equorum</i>	RE2.35	Se_RE2.35	Rice seed	India	PRJNA316783	SAMN04590070	LWJU000000000	98	2687155	586	Chaudhry <i>et al.</i> (direct submission)
<i>S. equorum</i>	RE2.24	Se_RE2.24	Rice seed	India	PRJNA316783	SAMN04590068	LWJS000000000	142	2874390	505	Chaudhry <i>et al.</i> (direct submission)
<i>S. equorum</i>	RE2.40	Se_RE2.40	Rice seed	India	PRJNA316783	SAMN04590072	LWJW000000000	91	2783269	613	Chaudhry <i>et al.</i> (direct submission)
<i>S. equorum</i>	SNUC 2835	Se_SNUC_2835	Bovine	Canada	PRJNA342349	SAMN06173018	QXSF000000000	194	2749917	212	Naushad (direct submission)
<i>S. equorum</i>	SNUC 3944	Se_SNUC_3944	Bovine	Canada	PRJNA342349	SAMN06173019	PYYI000000000	313	2920986	380	Naushad (direct submission)
<i>S. equorum</i>	SNUC 1644	Se_SNUC_1644	Bovine	Canada	PRJNA342349	SAMN06173015	PYYK000000000	303	2874640	204	Naushad (direct submission)
<i>S. equorum</i>	SNUC 2121	Se_SNUC_2121	Bovine	Canada	PRJNA342349	SAMN06173017	PYYJ000000000	337	2789163	202	Naushad (direct submission)
<i>S. equorum</i>	SNUC 5474	Se_SNUC_5474	Bovine	Canada	PRJNA342349	SAMN06173021	PYYG000000000	113	2730474	212	Naushad (direct submission)
<i>S. equorum</i>	SNUC 4019	Se_SNUC_4019	Bovine	Canada	PRJNA342349	SAMN06173020	PYYH000000000	163	2719872	386	Naushad (direct submission)
<i>S. equorum</i>	SNUC 115	Se_SNUC_115	Bovine	Canada	PRJNA342349	SAMN06173006	QXSK000000000	197	2797521	212	Naushad (direct submission)
<i>S. equorum</i>	SNUC 1937	Se_SNUC_1937	Bovine	Canada	PRJNA342349	SAMN06173016	QXSG000000000	172	2738866	207	Naushad (direct submission)
<i>S. equorum</i>	SNUC 1023	Se_SNUC_1023	Bovine	Canada	PRJNA342349	SAMN06173014	QXSH000000000	184	2709348	203	Naushad (direct submission)
<i>S. equorum</i>	SNUC 5082	Se_SNUC_5082	Bovine	Canada	PRJNA342349	SAMN06173022	PYYF000000000	49	2765966	207	Naushad (direct submission)
<i>S. equorum</i>	SNUC 742	Se_SNUC_742	Bovine	Canada	PRJNA342349	SAMN06173013	PYYL000000000	163	2707883	213	Naushad (direct submission)
<i>S. equorum</i>	SNUC 144	Se_SNUC_144	Bovine	Canada	PRJNA342349	SAMN06173008	PYYO000000000	157	2707401	225	Naushad (direct submission)
<i>S. equorum</i>	SNUC 476	Se_SNUC_476	Bovine	Canada	PRJNA342349	SAMN06173011	PYYM000000000	319	2879564	209	Naushad (direct submission)
<i>S. equorum</i>	SNUC 193	Se_SNUC_193	Bovine	Canada	PRJNA342349	SAMN06173009	PYYN000000000	66	2660622	209	Naushad (direct submission)
<i>S. equorum</i>	SNUC 116	Se_SNUC_116	Bovine	Canada	PRJNA342349	SAMN06173007	PYYP000000000	151	2767656	205	Naushad (direct submission)
<i>S. equorum</i>	SNUC 614	Se_SNUC_614	Bovine	Canada	PRJNA342349	SAMN06173012	QXSI000000000	158	2759429	206	Naushad (direct submission)
<i>S. equorum</i>	NCTC12414	Se_NCTC12414	Horse skin	Belgium	PRJEB6403	SAMEA103980427	UHD101000001	4	2758007	6077	Schleifer <i>et al.</i> (direct submission)
<i>S. equorum equorum</i>	G8HB1	Se_G8HB1	Human associated	India	PRJNA275686	SAMN03352179	LAKE000000000	22	2799869	507	Nair <i>et al.</i> (direct submission)
<i>S. equorum</i>	AR8-13	Se_AR8_13	Insect	China	PRJNA338661	SAMN05560422	MDJP000000000	64	2746763	504	Pei <i>et al.</i> (direct submission)

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Table S5. Metadata of 74 cheese rind metagenomes used to map the halophilic and halotolerant genomes selected in this study.

This study ID	Cheese Technology	RindType	Country	Region or State	Animal	Sequencing Technology	Accession	Reference
1	Lactic paste	Washed	France	Bourgogne Champagne	Cow	Illumina	SRR12103075	This study
2	Lactic paste	Washed	France	Bourgogne Champagne	Cow	Illumina	SRR12103074	This study
3	Unpressed uncooked soft	Washed	France	Alsace-Lorraine	Cow	Illumina	SRR12103073	This study
4	Pressed uncooked soft	Washed	France	Savoie	Cow	Illumina	SRR12103072	This study
5	Pressed uncooked semihard	Washed	France	Aquitane Midi-Pyrénées	Ewe	Illumina	SRR12103071	This study
6	Lactic paste	Washed	France	Méditerranée	Ewe	Illumina	SRR12103070	This study
7	Blue cheese	Natural	France	Auvergne	Cow	Illumina	SRR12103069	This study
8	Pressed uncooked soft	Natural	France	Auvergne	Cow	Illumina	SRR12103068	This study
9	Pressed uncooked soft	Natural	France	Franche-Comté	Cow	Illumina	SRR12103067	This study
10	Lactic paste	Bloomy	France	Méditerranée	Goat	Illumina	SRR12103066	This study
11	Unknown	Natural	USA	Connecticut Burgundy-Champagne	Cow	Illumina	4524482.3	1
12	Unknown	Washed	France	Savoie	Cow	Illumina	4524483.3	1
13	Unknown	Bloomy	France	Savoie	Goat	Illumina	4524484.3	1
14	Unknown	Bloomy	France	Normandy	Cow	Illumina	4524485.3	1
15	Unknown	Washed	Ireland	Co._Cork	Cow	Illumina	4524486.3	1
16	Unknown	Washed	France	Doubs_county	Cow	Illumina	4524487.3	1
17	Unknown	Natural	England	Nottinghamshire	Cow	Illumina	4524488.3	1
18	Unknown	Bloomy	England	Somerset	Goat	Illumina	4524489.3	1
19	Unknown	Natural	USA	Vermont	Cow	Illumina	4524490.3	1
20	Unknown	Natural	USA	Vermont	Cow	Illumina	4524491.3	1
21	Unknown	Natural	USA	Massachusetts	Cow	Illumina	4524493.3	1
22	Unknown	Washed	USA	California	Cow	Illumina	4524494.3	1
23	Unknown	Washed	USA	Virginia	Cow	Illumina	4524495.3	1
24	Unknown	Washed	France	Auvergne	Cow	Illumina	4524496.3	1
25	Unknown	Natural	France	Auvergne	Cow	Illumina	4524497.3	1
26	Unknown	Natural	Spain	Catalunya	Goat	Illumina	4524498.3	1
27	Unknown	Washed	USA	Vermont	Cow	Illumina	4524499.3	1
28	Unknown	Washed	Switzerland	Tufertschwil	Cow	Illumina	4524500.3	1
29	Unknown	Natural	USA	Vermont	Goat	Illumina	4524501.3	1
30	Unknown	Washed	France	Rhone-Alpes	Cow	Illumina	4524502.3	1
31	Unknown	Bloomy	Italy	Piedmont	Sheep	Illumina	4524504.3	1
32	Unknown	Washed	USA	Wisconsin	Cow	Illumina	4524505.3	1
33	Blue cheese	Natural	France	Auvergne	Cow	SOLiD	SAMEA7202606	This study
34	Blue cheese	Natural	France	Auvergne	Cow	SOLiD	SAMEA7202608	This study
35	Blue cheese	Natural	France	Midi-Pyrenees	Cow	SOLiD	SAMEA7202610	This study
36	Blue cheese	Natural	France	Auvergne	Cow	SOLiD	SAMEA7202612	This study
37	Blue cheese	Natural	France	Auvergne	Cow	SOLiD	SAMEA7202614	This study
38	Blue cheese	Natural	Italy	Lombardia	Cow	SOLiD	SAMEA7202617	This study
39	Blue cheese	Natural	Italy	Lombardia	Cow	SOLiD	ERS627441	2
40	Blue cheese	Natural	France	Midi-Pyrenees	Ewe	SOLiD	SAMEA7202618	This study
41	Unpressed uncooked soft	Bloomy	France	Brie	Cow	SOLiD	SAMEA7202619	This study
42	Unpressed uncooked soft	Bloomy	France	Brie	Cow	SOLiD	SAMEA7202620	This study
43	Unpressed uncooked soft	Bloomy	France	Brie	Cow	SOLiD	SAMEA7202621	This study
44	Unpressed uncooked soft	Bloomy	France	Normandie	Cow	SOLiD	SAMEA7202623	This study
45	Unpressed uncooked soft	Bloomy	France	Normandie	Cow	SOLiD	SAMEA7202625	This study

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46	Unpressed uncooked soft	Bloomy	France	Champagne	Cow	SOLiD	SAMEA7202626	This study
47	Unpressed uncooked soft	Bloomy	France	Normandie	Cow	SOLiD	SAMEA7202627	This study
48	Unpressed uncooked soft	Bloomy	France	Poitou	Goat	SOLiD	SAMEA7202628	This study
49	Unpressed uncooked soft	Bloomy	France	Centre-Val de Loire	Goat	SOLiD	SAMEA7202629	This study
50	Lactic paste Unpressed uncooked soft	Washed	France	Bourgogne Champagne	Cow	SOLiD	ERS627440	2
51	Unpressed uncooked soft	Washed	France	Alsace	Cow	SOLiD	SAMEA7202631	This study
52	Unpressed uncooked soft	Washed	France	Normandie	Cow	SOLiD	ERS627442	2
53	Pressed cooked hard	Natural	France	Savoie	Cow	SOLiD	SAMEA7202632	This study
54	Pressed cooked hard	Natural	France	Franche-Comté	Cow	SOLiD	SAMEA7202634	This study
55	Pressed cooked hard	Natural	France	Franche-Comté	Cow	SOLiD	SAMEA7202635	This study
56	Pressed cooked hard	Natural	France	Hauts-de-France	Cow	SOLiD	SAMEA7202636	This study
57	Pressed cooked hard	Natural	France	Hauts-de-France	Cow	SOLiD	SAMEA7202637	This study
58	Pressed cooked hard	Natural	Italy	Toscane	Cow	SOLiD	SAMEA7202638	This study
59	Pressed uncooked semihard	Natural	France	Auvergne	Cow	SOLiD	SAMEA7202639	This study
60	Pressed uncooked semihard	Natural	France	Auvergne	Cow	SOLiD	SAMEA7202640	This study
61	Pressed uncooked semihard	Natural	France	Aquitane Midi-Pyrenées	Ewe	SOLiD	SAMEA7202641	This study
62	Pressed uncooked semihard	Natural	France	Aquitane Midi-Pyrenées	Ewe	SOLiD	SAMEA7202642	This study
63	Pressed uncooked soft	Natural	Spain	Unknown	Cow	SOLiD	SAMEA7202644	This study
64	Pressed uncooked soft	Natural	France	Aveyron	Cow	SOLiD	SAMEA7202645	This study
65	Pressed uncooked soft	Natural	France	Franche-Comté	Cow	SOLiD	SAMEA7202646	This study
66	Pressed uncooked soft	Natural	France	Unknown	Cow	SOLiD	SAMEA7202647	This study
67	Pressed uncooked soft	Natural	France	Savoie	Cow	SOLiD	SAMEA7202648	This study
68	Pressed uncooked soft	Natural	France	Savoie	Cow	SOLiD	SAMEA7202649	This study
69	Pressed uncooked soft	Washed	France	Auvergne	Cow	SOLiD	SAMEA7202650	This study
70	Pressed uncooked soft	Washed	France	Auvergne	Cow	SOLiD	SAMEA7202652	This study
71	Pressed uncooked soft	Washed	France	Auvergne	Cow	SOLiD	SAMEA7202653	This study
72	Pressed uncooked semihard	Natural	France	Auvergne	Cow	SOLiD	SAMEA7202654	This study
73	Pressed uncooked semihard	Natural	France	Savoie	Cow	SOLiD	SAMEA7202655	This study
74	Pressed uncooked semihard	Natural	France	Savoie	Cow	SOLiD	SAMEA7202656	This study

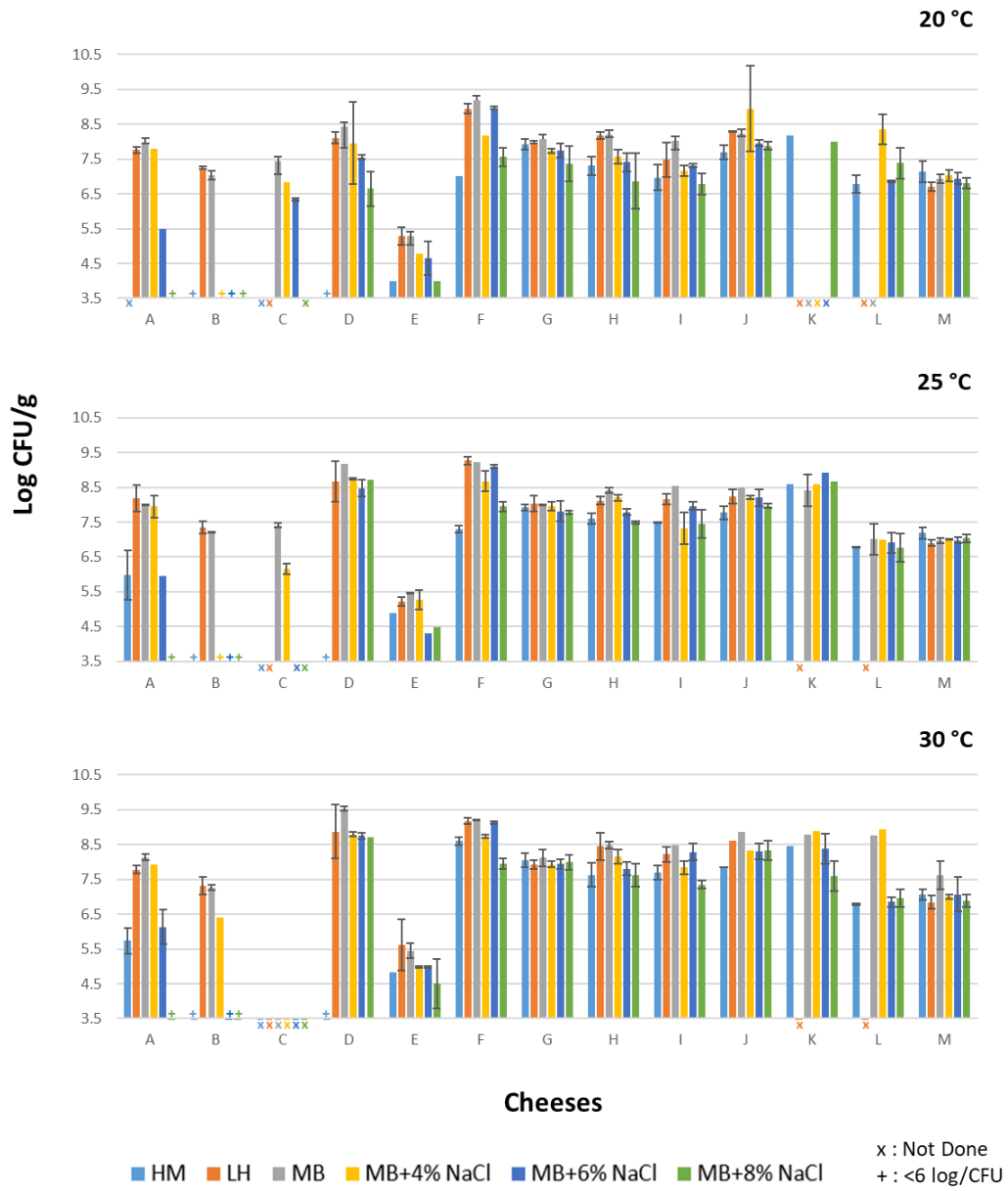


Figure S1. Total viable counts in different salt media and temperatures in log CFU/g from 13 cheese rind samples.

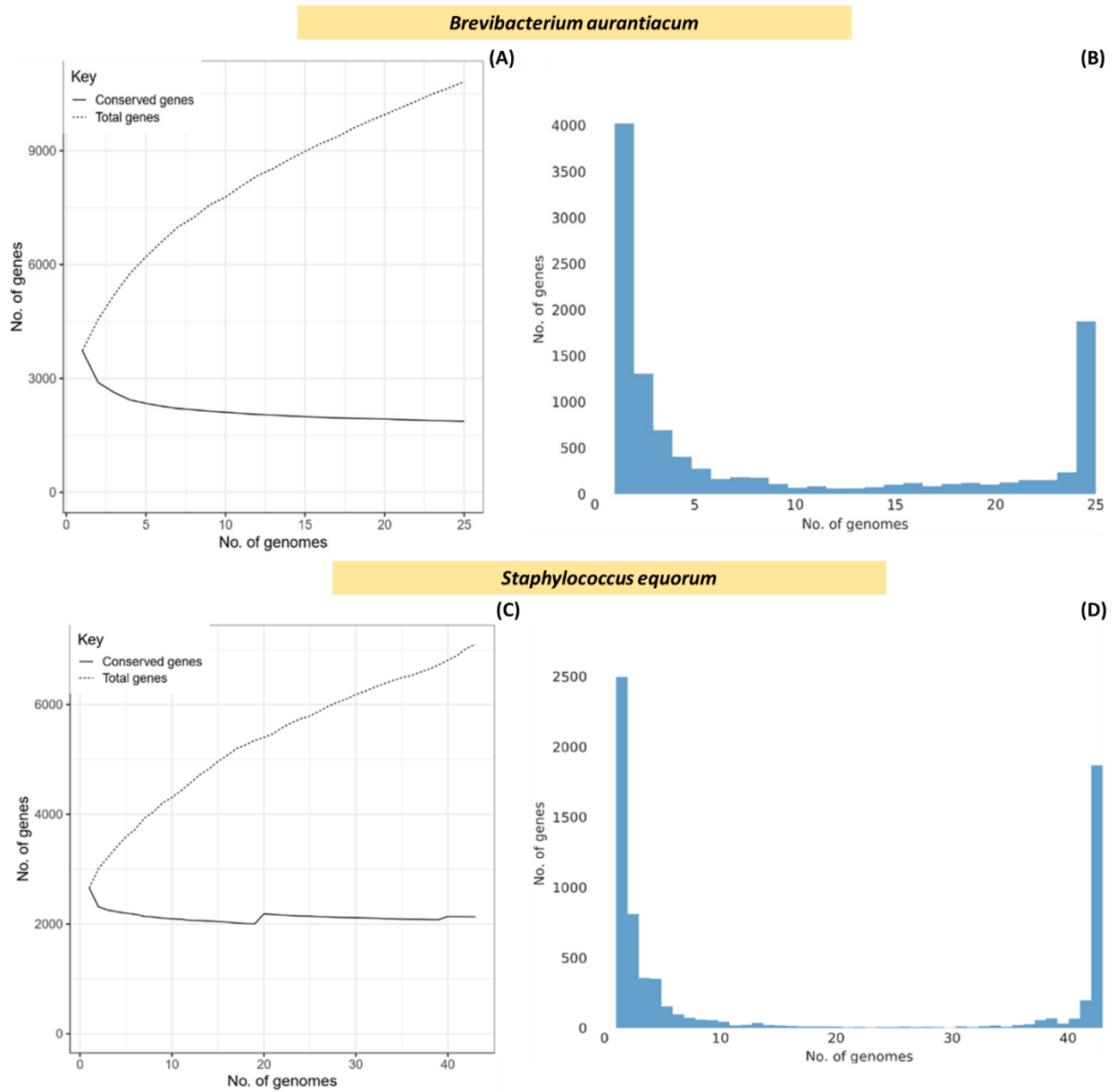


Figure S2. Pan- and core-genome size prediction with all combinations of 25 *B. aurantiacum* (A, B) and 43 *S. equorum* (C, D) strains and accumulated number of genes when an additional genome is introduced.

5 References

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1.3. Conclusion

Pour cette étude, nous avons séquencé 35 génomes bactériens, correspondant à 20 Gram-positifs et 15 Gram-négatifs. Parmi eux, les espèces Gram-positifs, les plus fréquemment isolées étaient les bactéries halotolérantes *Brevibacterium aurantiacum* (six souches) et *Staphylococcus equorum* (trois souches), qui sont, généralement, ajoutées comme ferments pour la production de fromages. Les analyses génomiques réalisées pour ces deux espèces confirment la grande diversité génétique entre les souches fromagères de *B. aurantiacum* et révèlent la présence de deux sous-espèces chez *S. equorum*, ainsi que la proximité phylogénétique de plusieurs souches de fromage avec des isolats bovins. Parmi les souches Gram-négatifs, nous avons identifié huit nouvelles espèces potentielles, en particulier aux genres *Halomonas* et *Psychrobacter*, ce qui confirme la faiblesse des études les concernant. De plus, le *mapping* des espèces halophiles et halotolérantes concernant 74 croûtes de fromage a montré que presque tous les échantillons, sauf cinq, possèdent dans leurs croûtes, ces bactéries tolérantes au sel. De tels résultats renforcent l'idée selon laquelle les bactéries halophiles jouent un rôle dans l'écosystème fromager. Il est intéressant de noter que les espèces Gram-négatifs d'*Halomonas*, *Pseudoalteromonas* et *Psychrobacter*, qui sont souvent associées en tant que contaminants, possèdent ensemble un niveau d'abondance supérieur à 10 % pour plusieurs métagénomomes de croûtes de fromage (n=18). Enfin, la disponibilité de ces isolats ouvre la possibilité d'explorer les caractéristiques physiologiques, ainsi que les propriétés fonctionnelles, potentielles, de ces bactéries dans le domaine de la technologie fromagère.

2. Stratégies pour isoler les espèces halophiles, rarement cultivées

2.1. Contexte

La diversité microbienne, à la surface des fromages, est étudiée depuis plusieurs décennies en appliquant des méthodes liées à la culture et, depuis la fin du 20^{ème} siècle, en utilisant des méthodes indépendantes de la culture, comme la métagénomique [5, 226]. Des études récentes, basées sur des technologies de séquençage à haut débit, ont démontré la grande diversité concernant les espèces halophiles dans les croûtes de fromage provenant de différents pays, européens et américains [3, 4]. À partir de ces résultats, et en observant l'absence d'isolats halophiles alimentaires dans la base de données (cf. la publication précédente), nous avons effectué un isolement systématique de ces souches, à partir de croûtes de fromage, en utilisant des techniques de culture, traditionnelles et touchant aussi différents milieux de culture à base de sel. Cette méthode a permis d'isoler une grande variété d'espèces, sans être très sélective. Cependant, l'accès à certains microorganismes halophiles par des méthodes de culture est encore peu développé et très limité dans les aliments, étant donné qu'ils peuvent être difficiles à cultiver. Cela peut justifier le manque constaté d'isolats halophiles alimentaires dans la littérature. Par conséquent, une partie de la diversité microbienne des fromages ne fait pas, jusqu'à présent, l'objet de cultures et des stratégies ciblées peuvent donc aider à leur isolement. Ainsi, les objectifs de cette étude consistent à :

- estimer la composition taxonomique des mêmes croûtes de fromages utilisées dans la [Publication 1](#) ;
- développer une stratégie ciblée pour isoler les espèces halophiles prédominantes non détectées lors de la première étude ;
- comprendre les possibles échecs ou difficultés affectant la culture de ces bactéries.

Les résultats obtenus sont présentés sous la forme d'un article en cours de préparation (cf. la partie 2.2).

2.2. Publication 2

Metagenomic driven characterization of halophilic bacteria in cheese rinds

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Abstract

Recent studies, based on high-throughput sequencing technologies, have demonstrated the abundance of poorly characterized halophilic bacteria on the surface of ripened cheeses. In this article, we investigated the results obtained by an extensive cultural study aiming the isolation of halophilic species, such as those belonging to the genera *Halomonas*, *Marinomonas*, *Pseudoalteromonas*, *Psychrobacter* and *Vibrio*, with metagenomic analyses. It indicated that number of species were not recovered previously, challenging a comprehensive recovery of these species. To achieve this task, we designed a strategy based on metagenomic-based probes, targeting the species of interest, coupled with an incremental cultural approach with pools. This strategy was performed on two cheese rinds from which many species were not isolated previously, targeting species displaying a relative abundance >3%, as calculated by coverage of the metagenomic assemblies. This method allowed the extensive isolation of targeted species, including potentially new cheese species, except two, which were dominant in each cheese with a relative abundance of 20.4 and 26.7%. These species corresponded to a new species of *Pseudoalteromonas*, without isolated representative yet, and to *Marinomonas foliarum*, isolated from marine environment. The possible reasons of their non-recovery were discussed with their metagenome-assembled genomes (MAGs) in comparison with closely related cultivable strains.

Keywords: food, targeted probes, genomic reconstruction, MAG, culture method

1 Introduction

In recent years, next-generation sequencing has been enabling the development of new approaches to study genetic diversity in microbial communities [1, 2]. Among them,

metagenomics is a powerful tool to analyze all microorganisms present in samples such as soil, sediments, food or water [3, 4]. This independent cultivation technique allows highlighting the existence of unexpected populations, including in already well-studied food ecosystems [5]. However, culture-independent techniques are unable to provide the biological material necessary to study the development of the ecosystems and its functionality [6].

The surface microbiota of cheese has been studied for several decades by conventional culture-based analysis and, since the end of the 20th century, by culture-independent methods [7, 8]. Recent studies, based on high-throughput sequencing technologies, have demonstrated a large species diversity and dominance of halophilic bacteria in several cheese rinds from different European and American countries [5, 9]. Based on these results and the poor availability of food halophilic isolates in the culture collections, Kothe *et al.* (2020) performed a systematic isolation of halophilic species from cheese rinds, using cultural techniques, on different salt-based media [10]. This method allowed the characterization of 26 species of halophilic and halotolerant species, including potentially ten undescribed species, which appeared to be frequent on cheese rinds, as measured by their genomic profiling. However, this analysis indicated that we failed to isolate all the existing species in each cheese, questioning the coverage of our analysis.

While profiling approach highlights species when reference genome is available, assembly-based metagenomics provides information independently of any reference set, at least for species whose abundance is enough to yield a good coverage [11-13]. Therefore, in this work, we carried out a metagenomic assembly-based analysis of ten previously studied cheeses and we showed that several abundant species had not been isolated previously. Consequently, we developed a fast and easy screening strategy guided by metagenomic assembly data to facilitate their isolation. As a proof of concept, we applied this strategy on two cheeses with important failure in our previous study with the aim to isolate their corresponding halophilic species.

2 Methodology

2.1 Cheese samples and taxonomic diversity

Ten cheese rind metagenomes, sequenced by Kothe *et al.* (2020) [10], were analyzed in this study (Table S1). For this purpose, we first estimated microbial community composition by mapping the samples reads against the representative clade-specific marker catalog contained on the MetaPhlAn tool v. 3.0.4 [14]. As an additional method for taxonomic profiling, we used a marker gene prediction, which allows binning of assembled metagenomic contigs without the need for reference sequence. Therefore, the reads were merged and *de novo* assembly was performed using SPAdes v. 3.9 [15]. Furthermore, genes were predicted using Prodigal v. 2.6.3 and the

marker genes were extracted using fetchMG v. 1.0 [16, 17]. By default, fetchMG searched for 40 universal marker genes. We focus our research on the COG0012 (ychF gene) and blasts were performed on the all-available sequences of the NCBI databases on nucleic and coding DNA sequences. Summary species composition plots were created in R v. 3.6.1 using the package ggplot2 v. 3.3.2.

2.2 Probe design and culture conditions

Species of two cheese rinds were chosen as proof of concept to build probes and for a subsequent targeted isolation. Briefly, we aligned the ychF sequences of interest species using ClustalX 2.1 [18] and we searched variable regions. The probes were designed with [Benchling Software](#) according to the following criteria: (i) primer size between 18-25 bp; (ii) product size between 90-700 bp; (iii) melting temperature (T_m) of both forward and reverse primers between 50-56°C; (iv) GC content of primers between 40-60%. The software [PCR Primer Stats](#) was applied to prevent the hairpins formations and self-annealing between them. The main features of the designed primers are presented in [Table 1](#).

Table 1. Characteristics of PCR primers designed to isolate the targeted species of two cheese samples.

	Closely species target (best blast)	Probe name	% reads	Amplicon size (bp)	Forward Primer Sequence	Reverse Primer Sequence
Cheese 9	<i>Psychrobacter cibarius</i>	A1	29.53	273	GTTAAGCCTGAGCGTGTATTACC	GGTTTGGTTGTGAATGGCAC
	<i>Marinomonas foliarum</i>	A2	26.73	440	CCTGGATGCTTTGGCTGAG	GAAGGTGTAGAGAGCGAACTTC
	<i>Psychrobacter translucens</i>	A3	16.36	364	GCCAGTACCAGATCCACGTC	GAACACATCTAGCATGGCTGC
	<i>Vibrio casei</i>	A4	10.70	204	GTTTCTAGCCGACTTAGGCATAG	ATAACTTCTGCACGGATGAAGC
	<i>Vibrio litoralis</i>	A5	4.28	657	CTACGTCTAGATGCGTTAGCTG	GTTTAGGCCTGGTTCTTCAATACC
	<i>Pseudoalteromonas</i> (unclassified)	A6*	3.61	383	GTTTGTGACATAGCAGGC	GAAAGTTAACGGAGGAAATCG
Cheese 10	<i>Pseudoalteromonas</i> (unclassified)	B1*	20.38	383	GTTTGTGACATAGCAGGC	GAAAGTTAACGGAGGAAATCG
	<i>Halomonas citrivedorans</i>	B2	8.71	477	TGAACGAGTCCTGCCTAC	AGGTAAGGGTTGTTCTCAAAC
	<i>Halomonas minus</i>	B3	8.48	90	ACGCTCTATGTTCTTAAGCG	GTTTGAAGGCCAATAATGAG
	<i>Pseudoalteromonas undina</i>	B4	5.06	636	ACGACATTGACGTGATCAAC	CGCTTTCGCCTTACAAG
	<i>Halomonas casei</i>	B5	3.81	540	CGACTTGATAAGCTTGCG	TTCTTCTGCCCAATCTC
	<i>Halomonas flavum</i>	B6	3.59	294	ATCCGCCATTGTAAAACCC	TTCACGGAGCGTAACAAG
	<i>Pseudoalteromonas nigrifaciens</i>	B7	2.56	294	ATCCGCCATTGTAAAACCC	TTCACGGAGCGTAACAAG

*Probe A6 = B1

All probes were checked in their corresponding cheese DNA in order to be a positive control. Then, the two cheese samples, collected by Kothe *et al.* (2020) [10], were homogenized in 0.9% NaCl sterile solution, diluted in series and spread on different salt-based agar media to display their cultivable cells. We tested the following media: Marine Agar (MA; Difco, Sparks, USA), MA added of 2, 4, 6 and 8% NaCl (Merck, Darmstadt, Germany), *Halomonas* Medium (HM; [19]) and Long and Hammer Agar (LH; [20]). The plates were incubated in a range of temperatures varying between 15 and 25°C.

Isolation were performed on plates where bacterial counts were comprised between 20 and 200 clones. Different morphotypes were selected and restreaked into a new plate to increase the amount of bacterial cells for fast DNA extraction. For this purpose, several colonies were mixed with a sterile loop in a tube containing 300 µl biomol water, 100 mg of 0.1 mm-diameter zirconium beads and 100 mg of 0.5 mm-diameter (Sigma, St. Louis, MO, USA) for their DNA extraction. The tube was agitated in a bead-beater (FastPrep-24, MP Biomedicals Europe, Illkirch, France) for 20 s at 4.5 m/s to release the DNA from the bacteria. The supernatant of this lysis was amplified using the targeted probes elaborated for each specific species. Thermal cycling conditions used were (i) 1 min at 94°C to initial denaturation, (ii) 30 cycles of 1 min at 94°C to denaturation, 0.5 min at 56°C to primer annealing, 1.5 min at 72°C to initial elongation, and (iii) 5 min at 72°C to final elongation. DNA amplification was checked on 0.8% agarose gel.

When the amplification signal was positive, the probable species corresponding was confirmed by sequencing its 16S rRNA gene. For this, we used the same thermal cycling conditions previously cited and the primers 27-F (5'-AGAGTTTGATCATGGCTCA-3') and 1492-R (5'-TACGGTTACCTTGTTACGACTT-3') [21]. Once received, the sequences were analyzed via NCBI BLAST tool [22] to obtain a taxonomic classification for each isolate. Finally, the extraction of genomic DNAs were performed using the protocol described by Almeida *et al.* (2014) [9], and their sequencing were carried out on the Illumina HiSeq platform at GATC-Biotech (Konstanz, Germany).

2.3. Metagenome-assembled genomes (MAGs)

The species unable to be cultivated, with the strategies used in this study, were reconstituted with MetaBAT2-2.12.1, an automated metagenomic binning tool [23], with minimum contig size of 1,500 nucleotides and other default settings. First, the genome binning was performed using the raw reads and the corresponding assembled-metagenomes. However, to improve the quality of MAGs, we also used a method assisted by the use of genome data of isolated strains in this study for each corresponding cheese (*Psychrobacter* FME60, FME61, *Vibrio* FME29 and FME62 for

Cheese 9, and *Halomonas* FME63, FME64, FME65, FME66, *Pseudoalteromonas* FME67 and FME68 for Cheese 10). Briefly, the reads of known genomes (isolated in this study or reliable sequences from database) were mapped against the raw reads of their corresponding metagenomes using Bowtie2-2.4.1 [24]. We filtered these reference genomes mapped, the remaining reads were assembled using SPAdes v. 3.9 [15] and new MAGs were recovered with MetaBAT2. The quality of the resulting bins were assessed with CheckM [25]. Finally, the presence of core genes from *Marinomonas foliarum* was also checked by read profiling using Bowtie2-2.4.1.

2.4 Genomic analyses

We performed genomic comparisons of the MAG strains with closely related genomes using the “Compare” and “Sequenced based” tools of SEED Viewer in RAST server v2.0 [26, 27]. The output was a table that includes the genes of the reference organism (MAGs) in chromosomal order and of the compared organisms correspondingly, with additional information such as the protein sequence identity (%) and if best-hits are bidirectional or unidirectional.

Additionally, prophage activity of all species obtained were annotated using Prophage Hunter [28], which is able to differentiate active from inactive prophages. Only active prophages were selected (region scoring > 0.8). [CRISPRCasFinder](#) was used with default parameters to discover CRISPR loci in halophilic genomes, predict CRISPR repeats and spacers and search for Cas proteins [29, 30].

2.5. Presence of new genomes on cheese rind metagenomes

The relative abundance of the halophilic genomes obtained in this study was determined in more than 90 cheese rind metagenomes from different countries and technologies, using the Food-Microbiome tool, as described previously [10]. The metadata of the samples are presented in [Table S2](#).

3 Results and Discussion

3.1 Evidencing absence of isolation of highly representative species by classical isolation procedures

The determination of the taxonomical composition of ten French cheese rinds with reference-based metagenomics tools, such as MetaPhlAn, indicates that several abundant species were not isolated during our previous work using cultural methods [10] (Table S3). For example, *Marinomonas foliarum* appears to be among the dominant species in Cheese 9, but have not been isolated. Furthermore, the pourcentage of reads aligned to the whole reference set was sometimes low, suggesting that several references were missing. Therefore, we employed a metagenomic reference-independent method based on assembly followed by marker gene analysis to point out all dominant species present in these cheeses. As results, 79 bacterial and three fungal species were identified in the ten cheese surface samples as shown Fig. 1 and Table S4. Overall, MetaPhlAn and the assembly-derived method gave similar results on number of species. However, marker gene analysis pointed the presence of several species belonging to the genus *Psychrobacter*, *Pseudoalteromonas*, *Halomonas*, *Pseudomonas* and *Corynebacterium*, which were not detected by MetaPhlAn. These species correspond mostly to halophiles, which are still poorly studied. For example, the Cheeses 9 and 10 contain an unclassified *Pseudoalteromonas* at estimated levels of 3.6 and 20%, repectively. Similarly, Cheeses 6, 7 and 9 contains the newly characterized species, *Psychrobacter translucens*, at levels of 2.7, 27 and 16%, respectively.

Finally, comparison of species isolated from the ten cheeses in our previous study [10] with the composition obtained by the marker analysis indicates that several species were not isolated (Fig. S1, hatched species). Therefore, we decided to develop targeted strategies to isolate strains corresponding to poorly characterized species in cheeses such as the still unclassified *Pseudoalteromonas* pointed out in Cheeses 9 and 10.

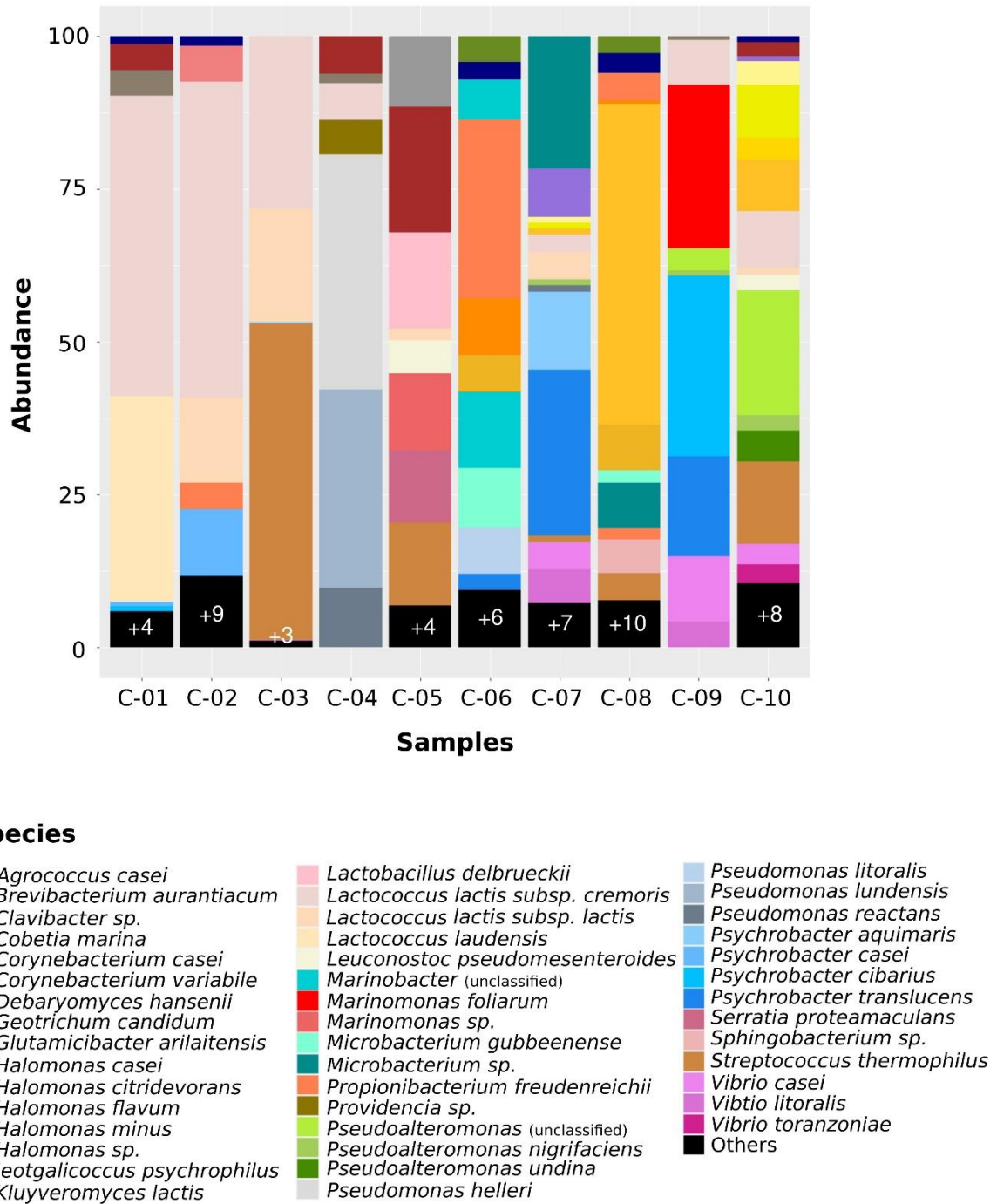


Figure 1. Taxonomic classification of microbial population in ten cheese rinds by marker gene analysis.

3.2 Strategy to target specific halophilic bacteria

In order to isolate the species missing in the previous cultural approach, some of which being less abundant or poorly cultivable, we defined a strategy based on a fast screening technique guided by the metagenomic analysis. The screening was based on the design of specific PCR primers to target the different species identified by the assembly-based metagenomic analysis. To design specific primers, metagenomic reads were assembled on contigs and sequences of marker genes were defined for each present species. Subsequently, a well-conserved gene, such as ribosome-binding ATPase YchF (COG0012), was selected to target any species. Therefore, their corresponding nucleotide sequences were aligned to define variable regions from which primers were designed to obtain pairs that allowed specific amplification for each species (Fig. 2A).

The above designed primers were finally tested on the metagenomic DNAs to confirm their efficiency. They can then be used to detect the presence of the targeted species through DNA extracted from single colony or from pools of colonies, as exemplified in Fig. 2B.

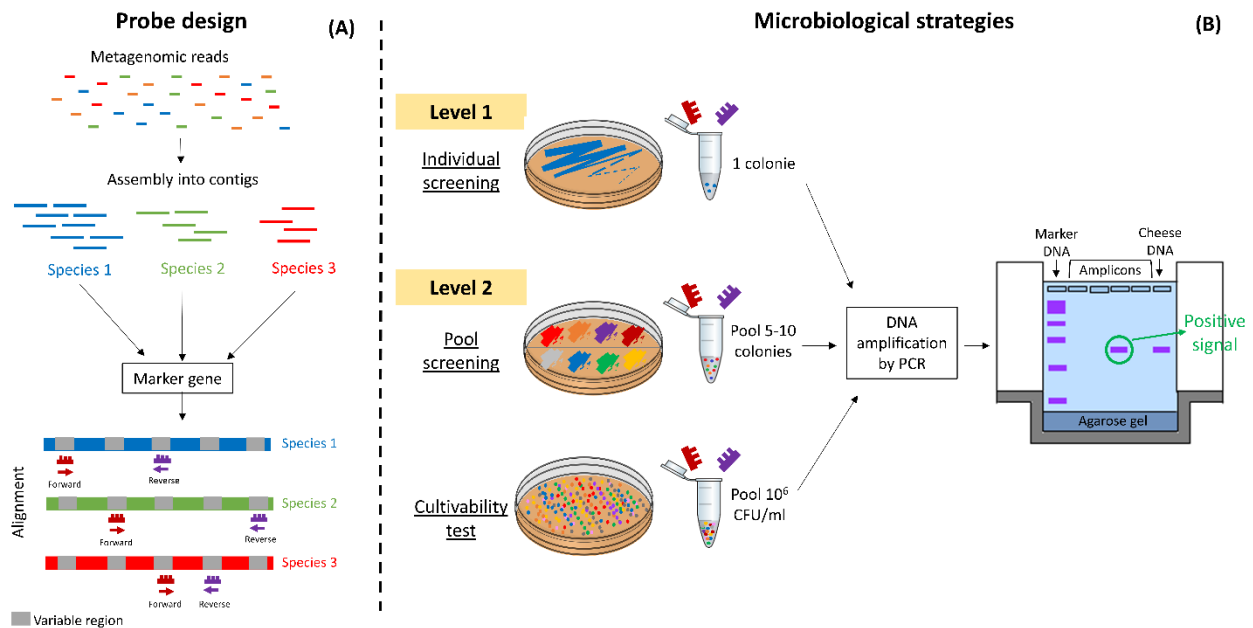


Figure 2. Probe design and culture strategies to isolate targeted species.

3.3 Targeted isolation of halophiles present in Cheeses 9 and 10

In this study, probes were designed to isolate halophilic bacteria present down to 2-3% from two cheeses, corresponding to six species of Cheese 9 and seven of Cheese 10 (Table 1). Interestingly, the dominant uncharacterized *Pseudoalteromonas* species in Cheese 10 was present as sub-dominant species in Cheese 9, as indicated by the 100% identity of their *ychF* nucleotide sequence (Probe A6 = B1).

In a first attempt, we applied an individual screening where colonies with different morphotypes (size, contour, relief, opacity, pigmentation and colony consistency) were picked and their DNA amplified with the different primers pairs. For those exhibiting a positive signal amplified, their identification was confirmed by 16S rRNA gene sequencing. Subsequently, for species of which no isolate was found in this first round, we developed a second screening attempt by testing pools of 5-10 mixed colonies, and by varying the culture conditions. The pools that exhibited a positive amplification, for a given primer pair, were separated and the clones were tested individually to isolate the targeted species. Finally, in case of negative result in this second attempt, we tested the cultivability of the targeted species on different medium and conditions in order to define suitable procedure for their isolation.

In this study, the first attempt was performed in MA plates at 25°C from which we isolated two out of the six targeted species in Cheese 9 (*Psy. cibarius* and *Psy. translucens* represented by the probes A1 and A3) and three out of the seven targeted species in Cheese 10 (*H. minus*, *H. casei* and *H. flavum* with B3, B5 and B6 probes; Table 2). Curiously, these species were not the most abundant in the cheese samples, as indicated in Fig. 1.

For the species that were not isolated in this first attempt, we used the second isolation procedure with pool strategies. Here, in addition to MA media, we also tested HM and LH media, which are frequently used to isolate environmental and food halophiles, as well as MA added of 2, 4, 6 and 8% NaCl. Considering the tendency of halophilic bacteria to be psychrophilic, we also tested temperatures of 15 and 20°C to expand the conditions to isolate them. By this way, we isolated *H. citridevorans* (B2) and *P. undina* (B4) from Cheese 10 on MA media at 25°C. Afterward, by using MA+4% of NaCl at 20 and 25°C, we isolated *V. casei* and *V. litoralis* (probes A4 and A5), respectively, from Cheese 9. Finally, still on MA+4% NaCl, but incubating at lower temperature (15°C), we isolated *P. nigrifaciens* from Cheese 10 (B7, Table 2).

Table 2. The ten isolates from this study and their respective strategy, media and temperature of isolation, and general features of their sequenced genomes.

	Targeted species	Probe	Strategy	Media	T (°C)	No of contigs	Size (base pairs)	GC content (%)	Genome accession
Cheese 9	<i>Psychrobacter cibarius</i> FME60	A1	morphotype	MA	25	85	3,469,065	42.6	JABUYU000000000
	<i>Psychrobacter translucens</i> FME61	A3	morphotype	MA	25	452	3,569,320	41.7	JABUYV000000000
	<i>Vibrio casei</i> FME29	A4	pool	MA+4%NaCl	20	115	3,833,673	40.5	JABUYT000000000
	<i>Vibrio litoralis</i> FME62	A5	pool	MA+4%NaCl	25	171	3,855,466	42.1	JABUYW000000000
Cheese 10	<i>Halomonas citridevorans</i> FME63	B2	pool	MA	25	77	3,927,099	54.2	JABUYX000000000
	<i>Halomonas minus</i> FME64	B3	morphotype	MA	25	48	3,871,780	53.8	JABUYZ000000000
	<i>Pseudoalteromonas undina</i> FME67	B4	pool	MA	25	127	4,193,916	40.1	JABWDP000000000
	<i>Halomonas casei</i> FME65	B5	morphotype	MA	25	143	4,336,586	53	JABUYZ000000000
	<i>Halomonas flavum</i> FME66	B6	morphotype	MA	25	122	4,506,877	54.2	JABUZA000000000
	<i>Pseudoalteromonas nigrifaciens</i> FME68	B7	pool	MA+4%NaCl	15	137	3,961,476	39.8	JABUZZ000000000

Despite the screening of over 2,000 colonies per cheese owing the use of pools, with different media and temperatures, we did not isolate *M. foliarum* co-dominant in Cheese 9 and the unclassified *Pseudoalteromonas*, which was the dominant species in Cheese 10 and sub-dominant in Cheese 9. In order to test the growth potential of these species under different conditions, we inoculated low dilution of cheese rind ($\sim 10^6$ UFC/mL) on agar plates using the different culture media mentioned above and incubating at 15, 20 and 25°C. However, we did not obtain any PCR signal after the extraction of DNA from these cultures, indicating that these microorganisms were not able to develop in these conditions and were overgrown by other strains. Interestingly, strains corresponding to *M. foliarum* and this unclassified species of *Pseudoalteromonas* have not yet been isolated from cheese. Indeed, growth of *Pseudoalteromonas* species often appear to be fastidious, suggesting that usual laboratory media are not well adapted to isolate these species [31]. However, *M. foliarum* was isolated from the seagrass *Posidonia oceanica* on Marine Agar, and was therefore expected to grow in our tests, especially since it represented about 30% of the initial population.

Finally, except these two species, we isolated all targeted species in Cheese 9 and 10, i.e. halophilic species which relative abundance is over ~3%, showing the efficiency of this approach to isolate sub-dominant bacteria. This method also indicates the cultivability of the different species.

3.3 Metagenome-assembled genome (MAG) and genomic analyses

In order to understand the isolation failures of *M. foliarum* and the unclassified *Pseudoalteromonas*, we reconstructed their genome sequences from the metagenomic data to compare them to closely related genomes of culturable strains. For this purpose, we applied several approaches including an automated metagenome binning software, MetaBAT, and a supervised method based on filters, taking advantage of our knowledge on the genome sequences of the other species isolated from the two cheeses. Indeed, such supervised method could be applied advantageously because (i) the two traced MAGs correspond to the dominant species with a high coverage and (ii) the sequences of the isolated strain appeared to correspond almost perfectly to those assembled in the metagenome. Finally, we obtained high quality MAGs for *M. foliarum* and the unclassified *Pseudoalteromonas*, with completeness of 100% and 93%, respectively, and contamination below 0.7% (Table 3).

Table 3. Genomic features are summarized for each metagenome-assembled genome (MAG) including number of contigs, size, GC content, and completeness and contamination estimation by CheckM.

Targeted species	Cheese	Method	No of contigs	Size (bp)	GC content (%)	Completeness (%)	Contamination (%)
<i>Marinomonas foliarum</i> FME69	9	Classical	115	3,700,451	43.5	95.49	0.00
		Filtered with reference genomes	117	3,776,195	43.5	100	0.21
<i>Pseudoalteromonas</i> (unclassified) FME70	10	Classical	176	3,450,867	39.7	91.21	1.06
		Filtered with reference genomes	190	3,434,613	39.7	93.27	0.68

Marinomonas foliarum MAG-FME69, obtained from Cheese 9, was compared with *M. foliarum* CECT 7731^T and *M. alcarazii* CECT 7730^T, which were isolated from the leaves of a marine plant [32], and with two closely related strains of *Marinomonas sp.* isolated from marine environment (MWYL1, [NC_009654](#)) and cheese (UCMA 3892) [33]. The two *M. foliarum* strains shares 97% ANI on over 80% of their genome size; while it fall to 85 and 80% with *M. alcarazii* and the two last strains, but still on over 60% of their size, indicating their phylogenetic closeness.

CECT 7731^T strain contains 3,980 genes against 3,651 for MAG-FME69, indicating its genome encodes slightly more genes, or that several genes were not recovered by metagenomic binning. Importantly, they share 3,158 orthologous genes, which represent around 80% of their genes. Bi-directional hit analysis between CECT 7731^T and MAG-FME69 genomes indicated that the environmental genome contains 750 specific genes against 493 of the cheese MAG. About 75% of CECT 7731^T specific genes were clustered in 38 islands (with over than 6 genes), and 50% annotated as “hypothetical protein” (Table S5). The main islands are (i) a potential plasmid (67 genes encoding dicarboxylic and lipid metabolic genes) (ii) phages (89 genes in four islands), (iii) dicarboxylic acids, sugar transport and metabolism (spread in 12 islands, totaling 128 genes), surface factors such as polysaccharides (one island with 26 genes). Additionally, three islands of 39, 37, and 33 genes are encoding significant metabolic functions. However, when compared with MWYL1, UCMA 38921 and CECT 7730^T strains, only 73 genes were missing in MAG-FME69, including two sugar assimilation systems, two histidine transport genes, two genes involved in glycogen synthesis and urease degradation genes. The absence of these genes could not explain growth defect of the cheese strain on the different tested media, which contain abundant nitrogen and sugar sources. Finally, the potential of the 493 specific genes of MAG-FME69 vs CECT 7731^T were searched and indicated the presence of at least three prophages, two plasmids and only few miscellaneous metabolic functions (Table S5). It contains also CRISPRs genes, including 185 spacers, showing an intense phage metabolism. In order to characterize eventual phage replications in the metagenomic assembly, we search for phage genes in contigs displaying higher coverage than MAG-FME69 (Table S5). From this analysis, we found integrated prophage genes from “*Moraxella*”, and interestingly, lactococcal or streptococcal phage genes at coverage of 68 and 444 against 18 for *L. lactis* genome, suggesting their replication. These data indicated that phages could be responsible of the lack of cultivability of this *Marinomonas* strain.

A similar analysis was performed with *Pseudoalteromonas* MAG-FME70 in comparison with *Pseudoalteromonas* sp. 3D05, *P. mariniglutinosa* KCTC 22327^T, *P. haloplanktis* ATCC 14393^T, which are among its closest environmental relatives (ANI ~77% on 47% of their length, and 62-68% of FME70 length), completed with two *Pseudoalteromonas* cheese strains (FME14 and JB197). The set of reference strains shared 2,456 orthologous genes, among which 244 were missing in MAG-FME70 (Table S6). An inspection of these genes indicated that MAG-FME70 strain do not contain genes encoding for ferric ion ABC transporter (3), cobalamine synthesis (3)

and agmatine-arginine pathway as well defined functions. Moreover, the main other functions potentially lacking in MAG-FME70 are linked to signal transduction (30), flagellar assembly (44) and transport system (12), completed by 54 hypothetical proteins.

Conversely, MAG-FME70 contains 680 specific genes compared to 3D05 strain (the closest relative strain), including primarily, 20 related to iron metabolism, 6 to sulfate metabolism, 11 for the purine/uric acid/allantoin catabolic super-pathway [34] and 15 for pili formation. Moreover, it contains 11 genes annotated as ton-dependent receptor, 29 as involved in the transport and 339 hypothetical proteins, among which were presented phage proteins (Table S6). Similar results were obtained with the MAG from the same species constructed in another study [11], suggesting that this list is robust, although it cannot be excluded that several of these genes may have been eliminated due to metagenomic procedure-dependent analysis. Overall, missing genes compared to other *Pseudoalteromonas* species could not sharply show a particular metabolic defect in the cheese strain that would completely impair its development. Nevertheless, it would be worth testing cobalamin supplementation, as well as addition of new nitrogen sources related to arginine/agmatine and allantoin/purine pathways and sulfate sources. Possible presence of bacteriophages was also tested in MAG-FME70. Nevertheless, while staphylococcal phage was detected at high level of coverage (about 20 times higher than *S. equorum* mean coverage), other phage genes potentially targeting Gammaproteobacteria were present on contig of comparable level of coverage than those of MAG-FME70, suggesting their docking in the chromosomal DNA (Table S6).

3.4 Representativeness of new genomes in cheese rinds

Finally, we analyzed the presence of these still uncultured species in cheeses by using their MAGs as genome reference, as well the other isolated species, on more than 90 cheese rind metagenomes from four studies (Table S7) [5, 10, 11, 35]. This analysis confirms a high relative abundance of species belonging to the genera *Psychrobacter*, *Halomonas* and *Pseudoalteromonas*, as previously demonstrated [10]. Concerning the MAGs recovered in this study, *Marinomonas foliarum* was present in only two additional cheese metagenomes evaluated with relative abundance of 2.6 and 4.4%. Additionally, the MAG of *Pseudoalteromonas* was present in seven other cheese rinds, two of which were highly represented (13.5 and 16.8%; Table S7).

4 Conclusion

Cultures provide a very biased view of the microbial composition of cheese. This fact is likely due to the different potential of revival of microorganisms once replicated after a long stationary phase to a new and different culture medium. In the present study, we showed a strategy to recover representative clones of each species present down to few percent's (2-3%), as measured by their DNA quantification. In particular, we isolated strains of *P. undina* and *V. litoralis*, which are, for our best knowledge, the first cheese isolates characterized yet. Nevertheless, we failed to isolate clones belonging to two species, both as part of the dominant species, according to DNA quantification. The first one, *M. foliarum*, have already been characterized from marine environment, and possess a phylogenetically closely related species isolated from cheese [32]. The second one was a *Pseudoalteromonas* species, which was not reported in culture collections neither in genome databases. The fact that the DNA sequence of both species could be assembled efficiently indicates that their DNAs were of good quality upon extraction and thus likely preserved in physically intact cells.

Metabolic features that explain their lack of growth were searched in their MAGs. In both cases, no direct missing genes could explain the growth defect of both strains in the media used. Consequently, the easiest explanation for their inability of revival would be their susceptibility to freezing. However, while relatively few metabolic features extracted from its MAG appears to distinguish the *M. foliarum* from its characterized relative isolated from environment, the *Pseudoalteromonas* MAG presented several features deserving further attention. The size of its genome may be significantly smaller than the average, including those isolated from cheeses, although MAG procedure likely underestimates its size. Interestingly, comparing MAG-FME70 to several *Pseudoalteromonas* species, a set of genes encoding metabolic pathways appeared absent in the MAG, while it contains specific genes that may express interesting metabolic abilities, such as allantoin/purine pathways. These original features open the challenge to design adapted media for its further characterization.

Finally, this work present a relevant strategy to isolate sets of representative strains present in a food ecosystem and serve as a basis for testing their role in the community and their impact on product development.

Supplementary material

The supplementary Table S1-S7 can be found at <http://dx.doi.org/10.17632/7vc3vndc3r.1>.

Table S1. Metadata of ten cheese analyzed in this study.

Sample	Cheese technology and origin	SampleName NCBI	Accession number	Biosample
C-01	Soft-ripened goat's milk cheese from Mediterranean region	Cheese_FME61	SRR12103066	SAMN15394673
C-02	Semi-soft blue cheese rind with unpasteurized milk from Auvergne-Rhone-Alpes region	Cheese_FME58	SRR12103069	SAMN15394670
C-03	Semi-hard ewe's milk cheese rind from Aquitaine Midi-Pyrenees region	Cheese_FME56	SRR12103071	SAMN15394668
C-04	Soft smear-ripened cheese rind from Champagne region	Cheese_FME53	SRR12103074	SAMN15394665
C-05	Semi-soft cheese rind from Savoie region	Cheese_FME55	SRR12103072	SAMN15394667
C-06	Semi-soft smear-ripened cheese rind with unpasteurized milk from Vosges-Alsace region	Cheese_FME54	SRR12103073	SAMN15394666
C-07	Soft ewe's milk cheese rind from Mediterranean region	Cheese_FME57	SRR12103070	SAMN15394669
C-08	Semi-soft cow's milk cheese rind from Franche-Comte region	Cheese_FME60	SRR12103067	SAMN15394672
C-09	Soft smear-ripened cheese rind from Bourgogne region	Cheese_FME52	SRR12103075	SAMN15394664
C-10	Semi-soft cow's milk cheese rind from Auvergne region	Cheese_FME59	SRR12103068	SAMN15394671

Table S2. Metadata of cheese metagenomes used in the mapping analysis.

Cheese ID	Cheese Technology	RindType	Country	Region or State	Animal	Sequencing Technology	Accession	Reference
1	Soft	Bloomy	France	Méditerranée	Goat	Illumina	SRR12103066	This study
2	Blue	Natural	France	Auvergne	Cow	Illumina	SRR12103069	This study
3	Semihard	Washed	France	Aquitaine Midi-Pyrenées	Ewe	Illumina	SRR12103071	This study
4	Soft	Washed	France	Bourgogne	Cow	Illumina	SRR12103074	This study
5	Soft	Washed	France	Champagne	Cow	Illumina	SRR12103072	This study
6	Soft	Washed	France	Savoie	Cow	Illumina	SRR12103073	This study
7	Soft	Washed	France	Alsace-Lorraine	Cow	Illumina	SRR12103075	This study
8	Soft	Washed	France	Méditerranée	Ewe	Illumina	SRR12103070	This study
9	Soft	Washed	France	Franche-Comté	Cow	Illumina	SRR12103067	This study
10	Soft	Washed	France	Bourgogne	Cow	Illumina	SRR12103075	This study
11	Unknown	Natural	USA	Champagne	Cow	Illumina	SRR12103068	This study
12	Unknown	Washed	USA	Auvergne	Cow	Illumina	4524482.3	1
13	Unknown	Washed	France	Connecticut	Cow	Illumina	4524483.3	1
14	Unknown	Bloomy	France	Burgundy-Champagne	Goat	Illumina	4524484.3	1
15	Unknown	Bloomy	France	Savoie	Cow	Illumina	4524485.3	1
16	Unknown	Washed	Ireland	Normandy	Cow	Illumina	4524486.3	1
17	Unknown	Washed	France	Co._Cork	Cow	Illumina	4524487.3	1
18	Unknown	Washed	France	Doubs_county	Cow	Illumina	4524488.3	1
19	Unknown	Natural	England	Nottinghamshire	Cow	Illumina	4524489.3	1
20	Unknown	Bloomy	England	Somerset	Goat	Illumina	4524490.3	1
21	Unknown	Natural	USA	Vermont	Cow	Illumina	4524491.3	1
22	Unknown	Natural	USA	Vermont	Cow	Illumina	4524493.3	1
23	Unknown	Washed	USA	Massachusetts	Cow	Illumina	4524494.3	1
24	Unknown	Washed	USA	California	Cow	Illumina	4524495.3	1
25	Unknown	Washed	USA	Virginia	Cow	Illumina	4524496.3	1

24	Unknown	Washed	France	Auvergne	Cow	Illumina	4524496.3	1
25	Unknown	Natural	France	Auvergne	Cow	Illumina	4524497.3	1
26	Unknown	Natural	Spain	Catalunya	Goat	Illumina	4524498.3	1
27	Unknown	Washed	USA	Vermont	Cow	Illumina	4524499.3	1
28	Unknown	Washed	Switzerland	Tufertschwil	Cow	Illumina	4524500.3	1
29	Unknown	Natural	USA	Vermont	Goat	Illumina	4524501.3	1
30	Unknown	Washed	France	Rhone-Alpes	Cow	Illumina	4524502.3	1
31	Unknown	Bloomy	Italy	Piedmont	Sheep	Illumina	4524504.3	1
32	Unknown	Washed	USA	Wisconsin	Cow	Illumina	4524505.3	1
33	Blue	Natural	France	Auvergne	Cow	SOLiD	SAMEA7202606	This study
34	Blue	Natural	France	Auvergne	Cow	SOLiD	SAMEA7202608	This study
35	Blue	Natural	France	Midi-Pyrenees	Cow	SOLiD	SAMEA7202610	This study
36	Blue	Natural	France	Auvergne	Cow	SOLiD	SAMEA7202612	This study
37	Blue	Natural	France	Auvergne	Cow	SOLiD	SAMEA7202614	This study
38	Blue	Natural	Italy	Lombardia	Cow	SOLiD	SAMEA7202617	This study
39	Blue	Natural	Italy	Lombardia	Cow	SOLiD	ERS627441	2
40	Blue	Natural	France	Midi-Pyrenees	Ewe	SOLiD	SAMEA7202618	This study
41	Soft	Bloomy	France	Brie	Cow	SOLiD	SAMEA7202619	This study
42	Soft	Bloomy	France	Brie	Cow	SOLiD	SAMEA7202620	This study
43	Soft	Bloomy	France	Brie	Cow	SOLiD	SAMEA7202621	This study
44	Soft	Bloomy	France	Normandie	Cow	SOLiD	SAMEA7202623	This study
45	Soft	Bloomy	France	Normandie	Cow	SOLiD	SAMEA7202625	This study
46	Soft	Bloomy	France	Champagne	Cow	SOLiD	SAMEA7202626	This study
47	Soft	Bloomy	France	Normandie	Cow	SOLiD	SAMEA7202627	This study
48	Soft	Bloomy	France	Poitou	Goat	SOLiD	SAMEA7202628	This study
49	Soft	Bloomy	France	Centre-Val de Loire	Goat	SOLiD	SAMEA7202629	This study
50	Soft	Washed	France	Bourgogne Champagne	Cow	SOLiD	ERS627440	2
51	Soft	Washed	France	Alsace	Cow	SOLiD	SAMEA7202631	This study
52	Soft	Washed	France	Normandie	Cow	SOLiD	ERS627442	2
53	Hard	Natural	France	Savoie	Cow	SOLiD	SAMEA7202632	This study
54	Hard	Natural	France	Frache-Comté	Cow	SOLiD	SAMEA7202634	This study
55	Hard	Natural	France	Frache-Comté	Cow	SOLiD	SAMEA7202635	This study
56	Hard	Natural	France	Hauts-de-France	Cow	SOLiD	SAMEA7202636	This study
57	Hard	Natural	France	Hauts-de-France	Cow	SOLiD	SAMEA7202637	This study
58	Hard	Natural	Italy	Toscane	Cow	SOLiD	SAMEA7202638	This study
59	Semihard	Natural	France	Auvergne	Cow	SOLiD	SAMEA7202639	This study
60	Semihard	Natural	France	Auvergne	Cow	SOLiD	SAMEA7202640	This study
61	Semihard	Natural	France	Aquitane Midi-Pyrenées	Ewe	SOLiD	SAMEA7202641	This study
62	Semihard	Natural	France	Aquitane Midi-Pyrenées	Ewe	SOLiD	SAMEA7202642	This study
63	Soft	Natural	Spain	Unknown	Cow	SOLiD	SAMEA7202644	This study
64	Soft	Natural	France	Aveyron	Cow	SOLiD	SAMEA7202645	This study
65	Soft	Natural	France	Frache-Comté	Cow	SOLiD	SAMEA7202646	This study
66	Soft	Natural	France	Unknown	Cow	SOLiD	SAMEA7202647	This study
67	Soft	Natural	France	Savoie	Cow	SOLiD	SAMEA7202648	This study
68	Soft	Natural	France	Savoie	Cow	SOLiD	SAMEA7202649	This study
69	Soft	Washed	France	Auvergne	Cow	SOLiD	SAMEA7202650	This study
70	Soft	Washed	France	Auvergne	Cow	SOLiD	SAMEA7202652	This study
71	Soft	Washed	France	Auvergne	Cow	SOLiD	SAMEA7202653	This study
72	Semihard	Natural	France	Auvergne	Cow	SOLiD	SAMEA7202654	This study

73	Semihard	Natural	France	Savoie	Cow	SOLiD	SAMEA7202655	This study
74	Semihard	Natural	France	Savoie	Cow	SOLiD	SAMEA7202656	This study
75	Soft	Unknown	Ireland	Unknown	Sheep	Illumina	ERS3466744	3
76	Soft	Bloomy	Ireland	Unknown	Cow	Illumina	ERS3466745	3
77	Semihard	Unknown	Ireland	Unknown	Cow	Illumina	ERS3466746	3
78	Semihard	Washed	Ireland	Unknown	Cow	Illumina	ERS3466771	3
79	Semihard	Washed	Ireland	Unknown	Cow	Illumina	ERS3466747	3
80	Soft	Unknown	Ireland	Unknown	Cow	Illumina	ERS3466772	3
81	Soft	Bloomy	Ireland	Unknown	Cow	Illumina	ERS3466773	3
82	Hard	Washed	Ireland	Unknown	Goat	Illumina	ERS3466748	3
83	Hard	Natural	Ireland	Unknown	Sheep	Illumina	ERS3466774	3
84	Semihard	Unknown	Ireland	Unknown	Cow	Illumina	ERS3466775	3
85	Soft	Natural	Ireland	Unknown	Cow	Illumina	ERS3466749	3
86	Soft	Washed	Ireland	Unknown	Cow	Illumina	ERS3466777	3
87	Soft	Bloomy	Ireland	Unknown	Cow	Illumina	ERS3466789	3
88	Soft	Natural	Ireland	Unknown	Cow	Illumina	ERS3466790	3
89	Semihard	Bloomy	Ireland	Unknown	Goat	Illumina	ERS3466791	3
90	Hard	Natural	Ireland	Unknown	Cow	Illumina	ERS3466792	3
91	Hard	Natural	Ireland	Unknown	Goat	Illumina	ERS3466794	3
92	Hard	Natural	Ireland	Unknown	Cow	Illumina	ERS3466750	3
93	Hard	Natural	Ireland	Unknown	Cow	Illumina	ERS3466751	3

Table S3. The relative abundance of microbial composition of ten French cheese metagenomes using MetaPhlAn.

Species	C-01	C-02	C-03	C-04	C-05	C-06	C-07	C-08	C-09	C-10
Actinomycetaceae_bacterium_sk1b4	0	0	0	0	0	0.29109	0	0	0	0
Actinomycetales_bacterium_JB111	0	0	0	0	0	0	0	0.35251	0	0
Agrococcus_casei	0	0	0	0	0	8.19255	0	8.34963	0	0
Corynebacterium_casei	0	0.00369	0	0	0	51.2108	0	15.4885	0	0
Corynebacterium_flavescens	0	0	0	0	0.25998	0	0	0	0	0
Corynebacterium_variabile	0	0	0	0	0	19.6526	0	0.61929	0	0
Brevibacterium_antiquum	0	0.202	0	0	0	0	0	0	0	0
Brevibacterium_aurantiacum	0.96757	1.7033	0.02625	0	0	4.23944	0.09189	4.91215	0	1.19903
Brevibacterium_linens	1.48648	2.25694	0.00925	0	0	4.25165	0.06659	5.15942	0	1.09536
Brevibacterium_sp_239c	0.14179	0.03488	0	0	0	0	0	0	0	0
Brachybacterium_alimentarium	8.5708	0.15666	0.02215	0	0	0.02724	0.0338	0	0	0
Leucobacter_sp_G161	0	0	0	0	0	0.05465	0	0	0	0.38738
Arthrobacter_rhombi	0.44266	0	0	0	0	0	0	0	0	0
Glutamicibacter_arilaitensis	0	0	0.17068	0	0	2.15304	21.4939	1.65116	0	2.15174
Glutamicibacter_sp	0	0	0.06487	0	0	1.51103	16.6753	0.09404	0	2.28822
Glutamicibacter_sp_BW77	0	0	0	0	0	0	0	0.22941	0	0
Propionibacterium_freudenreichii	0	11.0583	0	0	0.87758	2.65119	0	6.18512	0	0
Psychroflexus_halocasei	0	0	0	0	0	0.06859	0	0	0	0.19547
Sphingobacterium_sp_JB170	0	0	0	0	0	0.73966	0	0.01869	0	0
Staphylococcus_equorum	0.10597	6.31793	0.004	0	0	0.34662	0.31234	0.44169	0	3.47745
Staphylococcus_succinus	0	0	0	0	1.36185	0	0.00499	0	0	0
Staphylococcus_vitulinus	0	0.13625	0	0	0	0.00257	0.41267	0	0	0
Staphylococcus_warneri	0	0	0	0	0	0	0	0	1.01311	0
Aerococcaceae_bacterium	0	0.00112	0	0	0	0.24194	0	0.07455	0	0

Alkalibacterium_gilvum	0	0.0038	0	0	0	0.00175	0	0.10198	0	0
Carnobacterium_maltaromaticum	0.12848	0.45563	0	0	0	0	0	0	0	0
Marinilactibacillus_psychrotolerans	0	0	0	0	0	1.04585	0.37135	0.71485	0	0
Enterococcus_faecalis	0.1171	0.00576	0	0.15746	0.57712	0	0	0	0	0.37182
Enterococcus_malodoratus	0	0	0	0.00849	1.29636	0	0	0.0235	0	0
Lactobacillus_brevis	0	1.5744	0	0	0	0.03413	0	0	0	0
Lactobacillus_curvatus	0	3.48061	0	0	0	0.61776	0	0.11868	0	0
Lactobacillus_delbrueckii	0.05914	0	0	0	31.1279	0	0	0.60611	0	0
Lactobacillus_helveticus	0.19328	0	0	0	0	0	0	0	0	0
Lactobacillus_nodensis	0	0.21142	0	0	0	0	0	0	0	0
Lactobacillus_parabuchneri	0	0.00352	0	0	0.55665	0.04258	0	0	0	0
Lactiplantibacillus_plantarum	0	7.02383	0	0	0	0	0	0.07707	0	0
Lactobacillus_rhamnosus	0	0	1.05034	0	0.17931	0	0	0	0	0
Leuconostoc_mesenteroides	6.086	0.43638	0	0.07074	4.24848	0	0.01118	0.16069	0.99066	0.33437
Leuconostoc_pseudomesenteroides	0.16573	0.62153	0	0.08176	15.9537	0	0	0	1.4621	6.67973
Lactococcus_garvieae	0	0	0	0	1.71479	0	0	0	0	0
Lactococcus_lactis	75.0842	64.0248	34.2544	2.66912	5.18375	0.41942	7.2111	2.04215	15.3166	11.1713
Lactococcus_petauri	0	0	0	0	6.18607	0	0	0	0	0
Lactococcus_raffinolactis	0.28672	0.04814	0	0	0	0	0	0	0	0
Streptococcus_parauberis	0	0	0	0	0.14246	0	0	0	0	0
Streptococcus_thermophilus	0.08563	0.23908	64.033	0	30.1165	0	1.2383	11.572	0	22.9015
Coprococcus_eutactus	0	0	0	0	0	0	0.01082	0	0.15969	0
Brucella_abortus	0	0	0.12591	0	0	0	0	0	0	0
Advenella_kashmirensis	0	0	0	0	0	0	0	0.30599	0	0
Advenella_sp_S44	0	0	0	0	0	0	0	3.31624	0	0
Pseudoalteromonas_nigrifaciens	0	0	0	0.01582	0	0.00807	0.39101	0	0.73761	2.05761
Pseudoalteromonas_sp_A601	0	0	0	0	0	0	0	0	0	0.10447
Pseudoalteromonas_tetraodonis	0	0	0	0.01459	0	0	0	0	0.00476	1.59606
Citrobacter_braakii	0	0	0	0	0	0	0.80788	0	0	0
Raoultella_terrigena	0	0	0	0.10152	0	0	0	0	0	0
Hafnia_paralvei	0	0	0	0.51781	0	0	0.19772	0	0	0
Morganella_psychrotolerans	0	0	0	0	0	0.10715	0	0.01083	0.26594	0
Ewingella_americana	0	0	0	0.85361	0	0	0.10766	0	0	0
Serratia_fonticola	0	0	0	1.12286	0	0	0	0	0	0
Serratia_grimesii	0	0	0	0	0	0	0	0	1.09529	0
Serratia_liquefaciens	0	0	0	0	0.20565	0	0.42422	0	0	0
Cobetia_marina	0	0	0.19187	0	0	0	38.1055	0	0.02608	0
Halomonas_campaniensis	0	0	0.00217	0	0	0.75616	1.68787	0	0	8.90725
Halomonas_sp_JB37	0	0	0.00099	0	0	0	1.50231	36.452	0	21.1857
Marinomonas_foliarum	0	0	0	0	0	0	0	0	30.4574	0
Actinobacillus_pleuropneumoniae	0	0	0	0.22591	0	0	0	0	0	0
Acinetobacter_johnsonii	0	0	0	0	0	0	0.81098	0	0	0
Psychrobacter_alimentarius	0	0	0	0	0	0	0.17587	0	0	0
Psychrobacter_immobilis	0.17503	0	0	0	0	0	0.22203	0	0	0
Psychrobacter_sp_JB385	0	0	0	0	0	0	0.14573	0	0	0
Psychrobacter_sp_JCM_18900	0	0	0	0	0	0	0.01392	0	0.16575	0
Pseudomonas_bauzanensis	0	0	0	0	0	1.33246	0	0	0	0
Pseudomonas_helleri	0	0	0	53.5006	0	0	0	0	0	0
Pseudomonas_lundensis	0	0	0	40.6597	0.01181	0	0	0	0	0
Pseudomonas_taetrolens	5.90346	0	0	0	0	0	0	0	0	0
Vibrio_casei	0	0	0.04408	0	0	0	7.47305	0.92178	48.305	8.05754
Vibrio_kanaloae	0	0	0	0	0	0	0	0	0	5.83791

Table S4. The relative abundance of microbial composition of ten French cheese metagenomes by marker gene analysis (ychF gene).

Species	C-01	C-02	C-03	C-04	C-05	C-06	C-07	C-08	C-09	C-10
Agrococcus casei	0	0	0	0	0	4.12969	0	2.69727	0	0
Leucobacter sp.	0	0	0	0	0	0	0	0.49041	0	0.96772
Microbacterium gubbeenense	0	0	0	0	0	9.84096	0	2.03521	0	0
Microbacterium sp.	0	0	0	0	0	0	0	7.41504	0	0
Pseudoclavibacter sp.	0	0	0	0	0	0	0	0.81408	0	0
Corynebacterium casei	0	0	0	0	0	29.2241	0	4.50689	0	0
Corynebacterium variabile	0	0	0	0	0	9.34891	0	0.55907	0	0
Corynebacterium xerosis	0	0	0	0	0	0	0.47494	1.25055	0	1.1385
Brachybacterium alimentarium	1.30828	0	0	0	0	0	0	0	0	0
Brevibacterium aurantiacum	1.22458	1.46187	0	0	0	2.95229	0	3.2073	0	0.95065
Clavibacter sp.	0	0	0	0	0	6.50206	0	0	0	0
Glutamicibacter ardleyensis	0	0	0	0	0	0	0	0.82389	0	0
Glutamicibacter arilaitensis	0	0	0	0	0	0	7.85112	0	0	0.79695
Propionibacterium freudenreichii	0	4.2812	0	0	0	0	0	1.83414	0	0
Sphingobacterium sp.	0	0	0	0	0	0	0	5.49262	0	0
Jeotgalicoccus psychrophilus	0	5.89971	0	0	0	0	0	0	0	0
Staphylococcus equorum	0	2.1406	0	0	0	0	0	0	0	1.53697
Atopostipes suicloacalis	0	0.65262	0	0	0	0	0	0	0	0
Carnobacterium maltaromaticum	0	0.41768	0	0	0	0	0	0	0	0
Marinilactibacillus psychrotolerans	0	0	0	0	0	1.31799	0	0.84841	0	0
Enterococcus malodoratus	0	0	0	0	1.21517	0	0	0	0	0
Enterococcus sp.	0	0.30543	0	0	0	0	0	0	0	0
Vagococcus sp.	0	0	0	0	0	3.11045	0.24232	0	0	0
Lactiplantibacillus plantarum	0	2.61049	0	0	0	0	0	0	0	0
Lactobacillus brevis	0	0.81447	0	0	0	0	0	0	0	0
Lactobacillus curvatus	0	1.63417	0	0	0	0	0	0	0	0
Lactobacillus delbrueckii	0	0	0	0	15.6889	0	0	0	0	0
Lactobacillus paracasei	0	0	0	0	0	0	0	0.53945	0	0
Lactobacillus rhamnosus	0	0	0.59496	0	0	0	0	0	0	0
Leuconostoc mesenteroides	1.33309	0.5221	0	0	2.14396	0	0	0	0	0
Leuconostoc pseudomesenteroides	0	0	0	0	5.45666	0	0	0	0	2.56162
Lactococcus garvieae	0	0	0	0	3.49071	0	0	0	0	0
Lactococcus lactis subsp. cremoris	49.1629	51.7529	28.2436	6.10034	0	0	2.85936	0	7.3713	9.22184
Lactococcus lactis subsp. lactis	0	13.94	18.4154	0	1.90402	0	4.45866	0.8337	0	1.19542
Lactococcus laudensis	33.5938	0	0	0	0	0	0	0	0	0
Lactococcus raffinolactis	0	2.55045	0	0	0	0	0	0	0	0
Streptococcus gallolyticus	0	0	0.31448	0	0	0	0	0	0	0
Streptococcus thermophilus	0	0	51.8068	0	13.6765	0	1.11467	4.46275	0	13.3774
Arenibacter (unclassified)	0	0	0	0	0	0	0	0.58849	0	0
Psychroflexus sp.	0	0	0	0	0	1.42343	0	0	0	1.08157
Advenella sp.	0	0	0	0	0	0	0	1.09852	0	0

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Marinobacter (unclassified)	0	0	0	0	0	12.4154	0	0	0	0
Pseudoalteromonas (unclassified)	0	0	0	0	0	0	0	0	3.6085	20.3791
Pseudoalteromonas nigrifaciens	0	0	0	0	0	0	1.01774	0	0.8524	2.56162
Pseudoalteromonas sp.	0	0	0	0	0	0	0	0	0	1.70775
Pseudoalteromonas undina	0	0	0	0	0	0	0	0	0	5.06632
Klebsiella michiganensis	0	0	0	0	0	0	0.82388	0	0	0
Kluyvera intermedia	0	0	0	0	0	0	1.0662	0	0	0
Proteus hauseri	0	0	0	0	0	1.441	0	0	0	0
Providencia heimbachae	0	0	0	0	0	0	0	0.49041	0	0
Providencia sp.	0	0	0	5.64509	0	0	0	0	0	0
Serratia proteamaculans	0	0	0	0	11.726	0	0	0	0	0
Cobetia marina	0	0	0	0	0	0	21.6148	0	0	0
Halomonas casei	0	0	0	0	0	0	1.0662	0	0	3.81397
Halomonas citrivedorans	0	0	0	0	0	0	0.87235	0	0	8.70951
Halomonas flavum	0	0	0	0	0	0	0	0	0	3.58627
Halomonas minus	0	0	0	0	0	0	0.96927	52.6016	0	8.48181
Halomonas sp.	0	0	0	0	0	6.00123	0	7.41013	0	0
Marinomonas foliarum	0	0	0	0	0	0	0	0	26.725	0
Marinomonas polaris	0	0	0	0	0	0	0	0	0	1.42312
Marinomonas sp.	0	0	0	0	12.6161	0	0	0	0	0
Acinetobacter sp.	0	0	0	0	0	0	0.46525	0	0	0
Psychrobacter aquimaris	0	0	0.25215	0	0	0	12.7944	0	0	0
Psychrobacter casei	0.76265	11.0163	0	0	0	0	0	0	0	0
Psychrobacter cibarius	0.78745	0	0	0	0	0	0	0	29.534	0
Psychrobacter pacificensis	0	0	0	0	0	0	1.35698	0	0	0
Psychrobacter sp.	1.90352	0	0.21674	0	0	0	2.81089	0	0	0
Psychrobacter translucens	0	0	0	0	0	2.70626	27.1397	0	16.358	0
Pseudomonas bauzanensis	0	0	0	0	0	2.07363	0	0	0	0
Pseudomonas helleri	0	0	0	38.423	0	0	0	0	0	0
Pseudomonas litoralis	0	0	0	0	0	7.51252	0	0	0	0
Pseudomonas lundensis	0	0	0	32.4137	0	0	0	0	0	0
Pseudomonas reactans	0	0	0	9.81517	0	0	1.0662	0	0	0
Pseudomonas sabulinigri	0	0	0	0	0	0	0	0	0	1.08157
Pseudomonas taetrolens	1.42609	0	0	0	0	0	0	0	0	0
Vibrio casei	0	0	0.15582	0	0	0	4.36173	0	10.7	3.35857
Vibrio litoralis	0	0	0	0	0	0	5.57333	0	4.2824	0
Vibrio sp.	0	0	0	0	0	0	0	0	0	1.5939
Vibrio toranzoniae	0	0	0	0	0	0	0	0	0	3.13087
Debaryomyces hansenii	0	0	0	0	11.4551	0	0	0	0	0
Geotrichum candidum	4.24107	0	0	6.10034	20.6269	0	0	0	0	2.277
Kluyveromyces lactis	4.25657	0	0	1.50232	0	0	0	0	0.5683	0

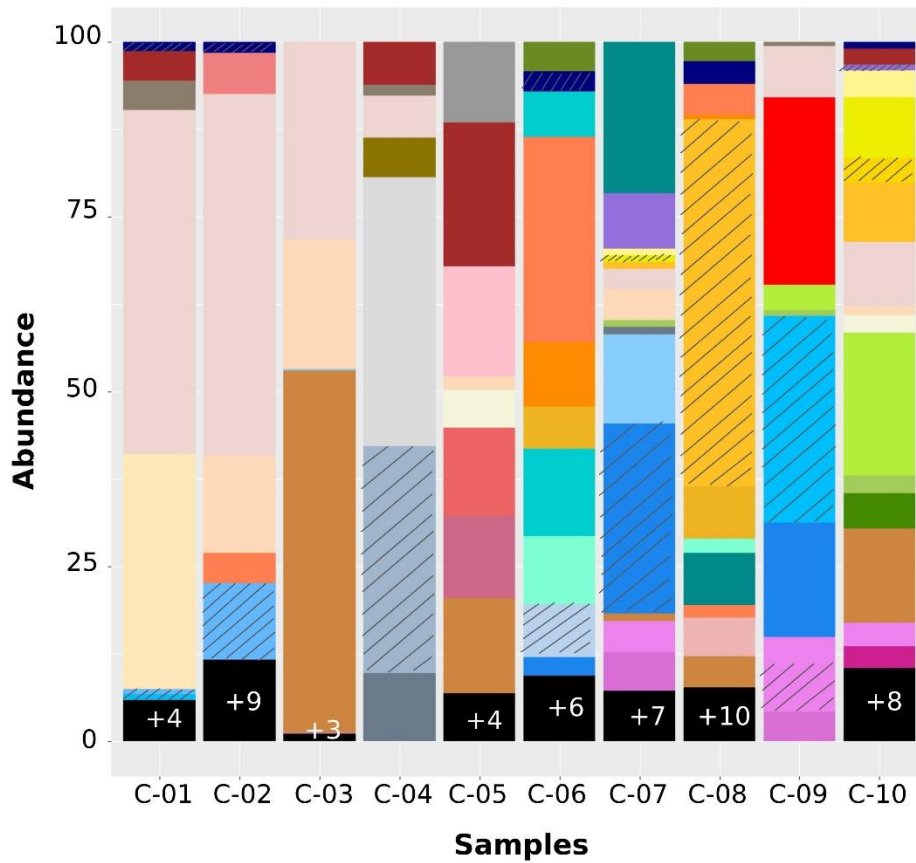
Table S5. Genomic analysis of *Marinomonas foliarum* MAG-FME69 and closely related cultivable species.

	<i>Marinomonas alcarazii</i> strain CECT 7730	<i>Marinomonas foliarum</i> strain CECT 7731	Marino_FME69_metabat_filter.bin.5.fa	<i>Marinomonas</i> sp. MWYL1	<i>Marinomonas</i> UCMA	Marino_CheeseSeq_R42_S43_bin5.fa	Marino_CheeseSeq_R46_S38_bin3.fa	Marino_CheeseSeq_R47_S50_bin7.fa	<i>Marinobacter hydrocarbonoclasticus</i> VT8
<i>Marinomonas alcarazii</i> strain CECT 7730	*	86.04 [76.65] 85.94 [74.24]	80.29 [75.84] 80.26 [75.43] 80.18 [72.44] 80.19 [75.03] 80.21 [74.69]	64.77 [13.14]					
<i>Marinomonas foliarum</i> strain CECT 7731	85.82 [73.86]	* 96.91 [79.05]	80.86 [71.87] 81.19 [73.44] 81.05 [71.12] 80.88 [71.76] 80.88 [71.52]	65.12 [13.09]					
Marino_FME69_metabat_filter.bin.5.fa	85.97 [78.55]	97.06 [87.38]	*	81.00 [77.01] 81.21 [78.16] 81.58 [77.60] 80.98 [76.36] 80.98 [76.12]	64.51 [12.67]				
<i>Marinomonas</i> sp. MWYL1	80.15 [61.07] 80.71 [60.25] 80.79 [57.78]		*	98.05 [86.33] 97.90 [79.76] 98.17 [83.88] 98.12 [83.55]	65.53 [10.99]				
<i>Marinomonas</i> UCMA	79.94 [60.65] 80.83 [61.89] 80.95 [59.22]		98.06 [86.65]	*	97.76 [82.43] 98.10 [85.63] 98.09 [85.13]	64.78 [10.71]			
Marino_CheeseSeq_R42_S43_bin.5.fa	79.95 [62.20] 80.72 [63.79] 81.19 [62.77]		97.96 [86.08] 97.75 [87.90]	*	98.12 [85.31] 98.09 [84.56]	64.49 [10.95]			
Marino_CheeseSeq_R46_S38_bin.3.fa	79.95 [63.27] 80.70 [62.99] 80.80 [60.50]		98.21 [89.56] 98.15 [89.95] 98.08 [84.20]	*	99.91 [98.01]	64.59 [10.98]			
Marino_CheeseSeq_R47_S50_bin.7.fa	79.80 [62.78] 80.54 [62.42] 80.62 [59.86]		98.02 [88.38] 97.94 [88.84] 97.88 [82.85]	99.73 [97.02]	*	64.65 [11.06]			
<i>Marinobacter hydrocarbonoclasticus</i> VT8	65.04 [11.31] 65.04 [11.82] 64.47 [10.54]		64.81 [11.19] 65.58 [12.47] 64.52 [10.75] 64.38 [10.83] 64.37 [10.81]	*					

Table S6. Genomic analysis of *Pseudoalteromonas* sp. MAG-FME70 and closely related species.

Table S7. Mapping of 12 species obtained in this study on 93 cheese rind metagenomes.

Species	C-09	C-10	C-11	C-12	C-22	C-23	C-24	C-25	C-50	C-77	C-85	C-86	C-87
<i>Vibrio litoralis</i> _FME62	3.98	0	0	0	0	0	0	0	3.62	0	3.41	0	0
<i>Vibrio casei</i> _FME29	8.55	2.33	0	5.01	2.12	0	0	0	0	0	0	0	2.62
<i>Pseudoalteromonas</i> sp._MAG-FME70	2.83	12.7	0	2.61	13.5	0	16.8	2.5	2.15	0.89	0	0	3.05
<i>Pseudoalteromonas undina</i> _FME67	0	5.54	0	0	0	0	0	0	0	0	0	0	0
<i>Pseudoalteromonas nigrifaciens</i> _FME68	0.94	4.16	0	12.7	23.2	0	0	0	2.28	0	16.5	0	4.36
<i>Marinomonas foliarum</i> _MAG-FME69	20.6	0	0	2.61	0	0	0	0	0	0	4.4	0	0
<i>Psychrobacter translucens</i> _FME61	15.8	0.16	0	0	2.61	0	2.71	0	0	0	0	0	11.8
<i>Psychrobacter cibarius</i> _FME60	26.2	0	0	0	0	0	1.19	0	5.52	0	0	0	0
<i>Halomonas minus</i> _FME64	0	6.92	0	0	0	0	20.5	0.84	0	0	0	0	0
<i>Halomonas flavum</i> _FME66	0	4.66	0	20.4	0	0	0	0	0	0	0	0	0
<i>Halomonas citridevorans</i> _FME63	0	6.45	0	14.2	11.6	0	0	0	0	0.79	0	0	6.67
<i>Halomonas casei</i> _FME65	0	3.41	0	0	0	0	5.85	0	0	0	0	0.42	0



Species

- | | | |
|-------------------------------------|---|-----------------------------------|
| <i>Agrococcus casei</i> | <i>Lactobacillus delbrueckii</i> | <i>Pseudomonas litoralis</i> |
| <i>Brevibacterium aurantiacum</i> | <i>Lactococcus lactis subsp. cremoris</i> | <i>Pseudomonas lundensis</i> |
| <i>Clavibacter sp.</i> | <i>Lactococcus lactis subsp. lactis</i> | <i>Pseudomonas reactans</i> |
| <i>Cobetia marina</i> | <i>Lactococcus laudensis</i> | <i>Psychrobacter aquimaris</i> |
| <i>Corynebacterium casei</i> | <i>Leuconostoc pseudomesenteroides</i> | <i>Psychrobacter casei</i> |
| <i>Corynebacterium variabile</i> | <i>Marinobacter (unclassified)</i> | <i>Psychrobacter cibarius</i> |
| <i>Debaryomyces hansenii</i> | <i>Marinomonas foliarum</i> | <i>Psychrobacter translucens</i> |
| <i>Geotrichum candidum</i> | <i>Marinomonas sp.</i> | <i>Serratia proteamaculans</i> |
| <i>Glutamicibacter arilaitensis</i> | <i>Microbacterium gubbeenense</i> | <i>Sphingobacterium sp.</i> |
| <i>Halomonas casei</i> | <i>Microbacterium sp.</i> | <i>Streptococcus thermophilus</i> |
| <i>Halomonas citridevorans</i> | <i>Propionibacterium freudenreichii</i> | <i>Vibrio casei</i> |
| <i>Halomonas flavum</i> | <i>Providencia sp.</i> | <i>Vibrio litoralis</i> |
| <i>Halomonas minus</i> | <i>Pseudoalteromonas (unclassified)</i> | <i>Vibrio toranzoniae</i> |
| <i>Halomonas sp.</i> | <i>Pseudoalteromonas nigrifaciens</i> | Others |
| <i>Jeotgalicoccus psychrophilus</i> | <i>Pseudoalteromonas undina</i> | |
| <i>Kluyveromyces lactis</i> | <i>Pseudomonas helleri</i> | |

Figure S1. Hatched species were isolated by Kothe *et al* (2020) [10].

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2.3. Conclusion

Au cours de cette étude, nous avons utilisé une stratégie ciblée pour l'isolement de bactéries halophiles, basée sur une technique de criblage rapide, guidée par l'analyse métagénomique, stratégie qui s'est avérée efficace. En bref, la détermination de la composition taxonomique de dix métagénomomes de croûtes de fromages a indiqué la prédominance de plusieurs espèces qui n'étaient pas isolées par des méthodes classiques ([Publication 1](#)). Pour isoler ces espèces manquantes, dont certaines sont potentiellement peu cultivables, nous avons défini une stratégie axée sur la conception d'amorces PCR spécifiques. Ainsi, deux fromages ont été choisis pour être incubés à différentes températures, dans des milieux de culture à base de sel ; et dans une première approche, un *screening* individuel de différents clones a été testé avec les amorces ciblées. Pour les espèces qui n'ont pas été isolées au cours de cette première phase, nous avons mis au point un deuxième niveau de criblage, basé sur le *pool* de cinq à dix colonies, augmentant ainsi la probabilité d'isoler ces espèces manquantes. Utilisant ces stratégies, nous avons pu isoler dix espèces présentes, avec des niveaux distincts d'abondance relative, dans deux fromages à croûte lavée. Cependant, nous avons échoué dans la culture de deux espèces représentées par une *Pseudoalteromonas* (nouvelle espèce) et une souche de *Marinomonas foliarum*, dominantes dans les fromages avec des *reads* métagénomiques de valeur respective 20 et 27%. Cherchant à comprendre le problème du non-isolement de ces souches, leurs MAGs ont été construits et comparés aux génomes, étroitement liés à des souches cultivables. Cependant, notre analyse génomique n'a pas révélé de différence notable entre les MAGs et les souches cultivables. Ainsi, l'hypothèse qui nous paraît la plus plausible réside dans le fait que les cellules bactériennes pourraient avoir été détruites par la congélation, sachant que les échantillons ont été conservés à une température de -20°C. Pour les travaux futurs, nous suggérons de tester, à la fois, leur isolement sans congeler les échantillons, et une gamme plus large de milieux de culture.

3. Description de nouvelles espèces

3.1. Contexte

Précédemment ([Publication 1](#) et [Publication 2](#)), nous avons isolé plusieurs souches à partir de croûtes de fromages qui, selon les analyses génomiques, représentent onze nouvelles espèces potentielles (ANI<95% avec des souches type d'espèces bien définies [227]). Elles comprennent une nouvelle espèce de chacun des genres suivants : *Advenella*, *Brachybacterium*, *Brevibacterium*, *Pseudomonas* et *Proteus* ; quatre nouvelles espèces d'*Halomonas* et deux de *Psychrobacter*. Comme les espèces Gram-négatifs d'*Halomonas* et de *Psychrobacter* étaient les plus nombreuses et avec un degré d'abondance élevé dans plusieurs croûtes de fromages, nous avons focalisé notre étude sur la proposition de nouvelles taxonomies d'espèces chez ces deux genres. De plus, pour augmenter la variabilité des souches fromagères au sein d'une même espèce, nous avons également analysé les isolats obtenus par Almeida *et al.* [4] et Wolfe *et al.* [3], représentant 12 souches d'*Halomonas* et quatre de *Psychrobacter*. Ainsi, cette étude représente une avancée en :

- démontrant, à travers l'analyse phylogénomique que ces isolats fromagers forment un clade d'espèces non encore décrit ;
- identifiant les caractéristiques physiologiques, enzymatiques et biochimiques particulières au sein de chaque nouvelle espèce étudiée ;
- proposant de nouvelles taxonomies (au niveau de l'espèce) pour les genres *Halomonas* et *Psychrobacter*.

Les résultats obtenus ont été présentés sous la forme de deux articles dans l'*International Journal of Systematic and Evolutionary Microbiology* ; ils constituent respectivement la partie 3.2 and 3.3 des nouvelles espèces d'*Halomonas* et *Psychrobacter*.

3.2. Publication 3

***Halomonas citridevorans* sp. nov., *Halomonas minus* sp. nov., *Halomonas casei* sp. nov. and *Halomonas flavum* sp. nov., four novel species isolated from French cheese rinds**

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Keywords: Taxonomy, sequence analysis, whole-genome sequencing, food.

Repositories:

The whole-genome and 16S gene sequences of the novel type strains of *Halomonas* have been deposited at DDBJ/ENA/GenBank and are available under the accession numbers JABUYX000000000, JABUYZ000000000 and JABUZA000000000, and MT573208, MT573212, MT573213 and MT573210, respectively.

ABSTRACT

Twelve Gram-negative bacterial strains were isolated from cheese rinds in France. On the basis of 16S rRNA gene sequence analysis, all isolates were assigned to the genus *Halomonas*, their closest relatives being *Halomonas alkaliphila*, *H. zhanjiangensis*, *H. hydrothermalis* and *H. venusta*, with similarities of 99.86, 99.78, 99.62 and 99.52%, respectively. Phylogenetic analyses of 16S rRNA and *rpoB* genes and average nucleotide identity (gANI), calculated using the complete genome sequences of the strains, revealed that they constituted four novel and well-supported clusters. The strains isolated herein differed from the previously described species by ANI < 95-96% and several biochemical, enzymatic and colony characteristics. In conclusion, the results of phenotypic and phylogenetic analyses indicated that the isolates belonged to four novel *Halomonas* species, for which the names *Halomonas citridevorans* sp.

nov., *Halomonas minus* sp. nov., *Halomonas casei* sp. nov. and *Halomonas flavum* sp. nov. are proposed, with isolates FME63^T (= CIRM-BIA2430^T = CIP 111880^T = LMG 32013^T), FME64^T (=CIRM-BIA2431^T = CIP 111877^T = LMG 32015^T), FME65^T (=CIRM-BIA2432^T = CIP 111881^T = LMG 32012^T) and FME66^T (=CIRM-BIA2433^T = CIP 111876^T = LMG 32014^T) as type strains, respectively.

Halomonas is a genus of the *Halomonadaceae* family that belongs to the *Oceanospirillales* order, *Gammaproteobacteria* class and *Proteobacteria* phylum [1, 2]. In total, 69 whole-genome sequences of defined *Halomonas* species were available at the time of writing (NCBI public database). Corresponding strains are predominantly halophilic [3, 4] and isolated from saline ecosystems such as soils, saline lakes and salterns [5, 6]. Some species were also recently described in fermented foods known to contain high salt concentrations, such as traditional Korean fermented seafood (*H. jeotgali*, *H. alimentaria* and *H. cibimaris*) [7-9], and in 'Quargel' cheese (*H. nigrificans*) [10].

In this paper, we describe 12 strains that belong to the *Halomonas* genus that were isolated from French cheese rinds. Since these strains differed by some unusual genetic and phenotypic features from the closely related *Halomonas* type strains, we established that they belong to four new *Halomonas* species referred to as *H. citrivedorans* sp. nov., *H. minus* sp. nov., *H. casei* sp. nov. and *H. flavum* sp. nov.

Isolation and ecology

During the course of several projects that explored the diversity of bacteria associated with artisanal cheese rinds, we isolated 12 strains that belong to potentially new *Halomonas* species. The sources of isolation and their corresponding growth media and temperature are presented in Table S1. Bacterial strains obtained from pure colonies were stored at -80°C in their respective growth media supplemented with 20% (v/v) glycerol and deposited in culture collection centers.

16S rRNA and *rpoB* phylogenies

Genomic DNA was extracted from the bacterial cells according to the protocol described by Almeida *et al.* (2014) [11]. Their 16S rRNA genes were amplified using 27-F (5'-AGAGTTTGATCATGGCTCA-3') and 1492-R (5'-TACGGTTACCTTGTTACGACTT-3') primers [12], and sequences were compared with those of type strains of species with validly published names using EzBioCloud (<https://www.ezbiocloud.net/>). Their accession numbers are presented in Table S2. In order to expand the taxonomy of strains, *rpoB* (DNA-directed RNA polymerase beta subunit), used as an alternative marker gene, was amplified using primers VIC4 (5'-GGCGAAATGGCDGARAACCA-3') and VIC6 (5'-GARTCYTCGAAGTGGTAACC-3') [13]. The *rpoB* sequences were compared to known bacterial species in the BLASTn GenBank database of the National Center for Biotechnology Information (NCBI). Further phylogenetic analyses were performed for 16S rRNA and *rpoB* sequences using MEGA 7.0.26 software, and trees were generated using the Neighbor-Joining algorithm with 1,000 bootstrap iterations [14] (Fig. 1 and Fig. 2). To delineate species, we used a threshold of above 99% identity for 16S rRNA genes with type strains [15], and above 97.7% identity for *rpoB* nucleotidic sequences [16].

The strains FME63^T, 1M45, JB380 and FME16 are closely related to each other, sharing identities ranging from 99.08 to 99.91% for 16S rRNA and from 97.25 to 100% for *rpoB* gene sequences (Table S3A). These strains could thus be representatives of a new genomospecies since their level of identity of 16S rRNA and *rpoB* with the closest related type strains is below the given thresholds. Moreover, the inferred phylogenetic trees confirmed the unambiguous divergence of the four strains with previously described *Halomonas* species, as well as the fact that they might form two distinct genomic groups (Fig. 1 and Fig. 2) that require further genomic analyses to confirm their correct assignment.

Similarly, analyses of 16S rRNA and *rpoB* genes indicated that FME64^T and FME1 belong to the same species since their sequences share identities of 100 and 98.14%, respectively. However, they remain unassigned since their sequences display sub-threshold levels of identity with all defined species (Table S3B).

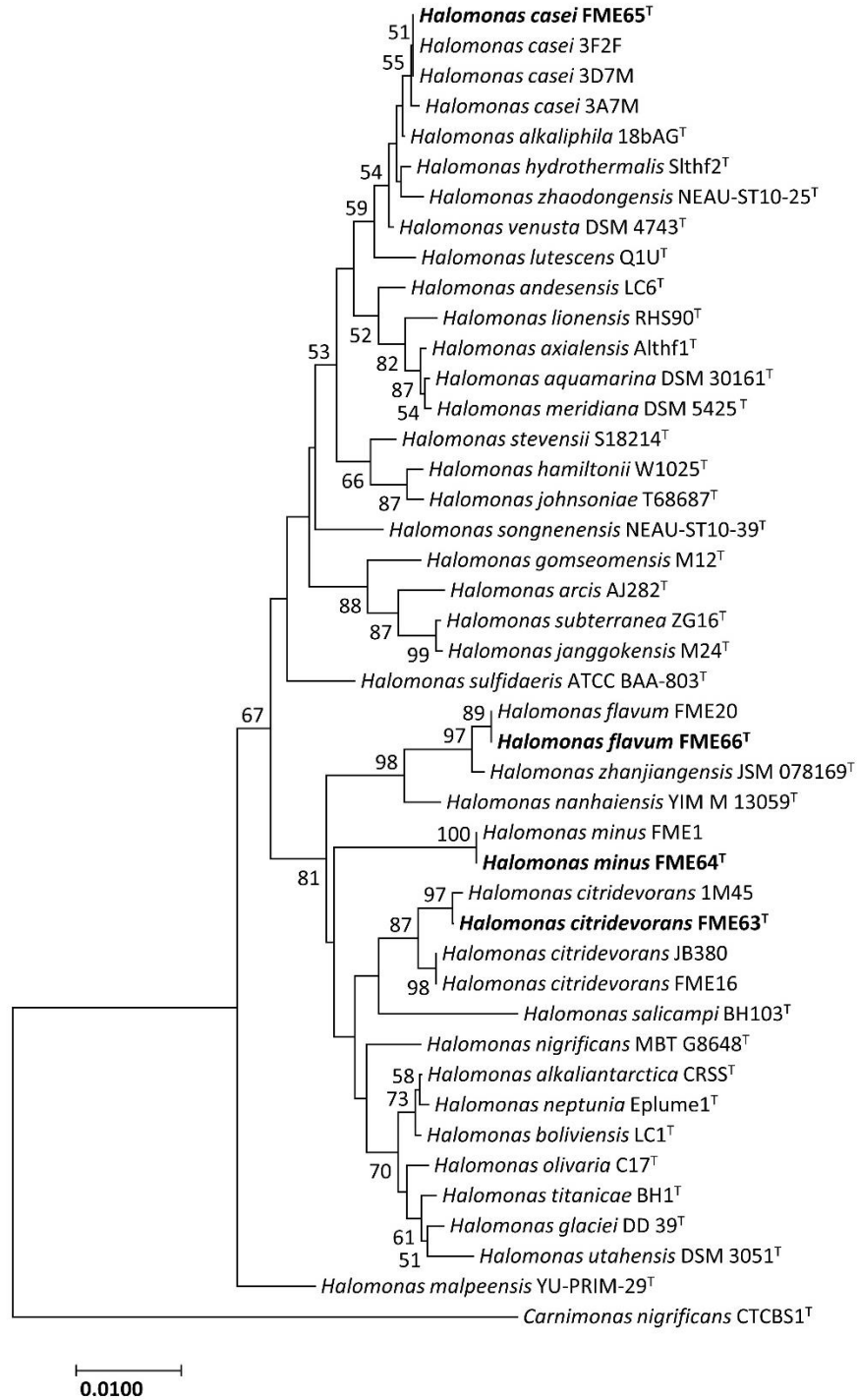


Figure 1. Phylogenetic tree based on 16S rRNA gene sequences of 12 strains from this study and closely related *Halomonas* type strains. The tree was built with MEGA 7.0.26 software using the neighbor-joining method. *Carnimonas nigrificans* CTCBS1^T was used as the outgroup.

The *Halomonas* strains - FME65^T, 3F2F, 3D7M and 3A7M - shared 16S rRNA and *rpoB* gene sequence identities >99.71 and 100%, respectively (Table S3C). They are also closely related to four environmental species, *H. alkaliphila* DSM 16354^T, *H. venusta* DSM 4743^T, *H. hydrothermalis* Slthf2^T and *H. zhaodongensis* NEAU-ST10-25^T, showing the highest similarity of between 99.27 and 99.86% for 16S rRNA sequences and of between 94.53 and 98.36% for *rpoB* sequences. This led to the assumption that these four strains isolated from cheese might be assigned to the species *H. alkaliphila*, but further analyses are required.

The 16S rRNA and *rpoB* gene sequences of *Halomonas* FME66^T and FME20 strains share 100 and 99.68% of homology with each other, respectively, and also display high identity (> 99.5% for 16S and > 98.5% for *rpoB*) with those of *Halomonas zhanjiangensis* JSM 078169^T (Table S3D). As a result, it was suggested that FME20 and FME66^T belong to this species, and the hypothesis that they form a new closely related species requires further investigations.

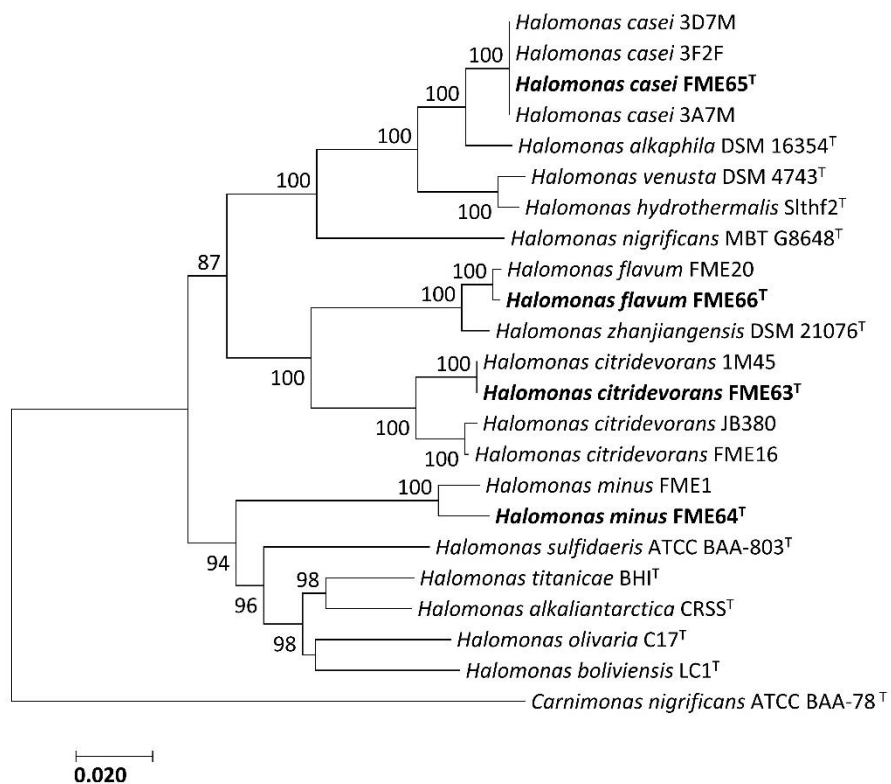


Figure 2. Phylogenetic tree based on *rpoB* gene sequences of 12 strains from this study and closely related *Halomonas* type strains. The tree was built with MEGA 7.0.26 software using the neighbor-joining method. *Carnimonas nigrificans* CTCBS1^T was used as the outgroup.

Genome features and phylogenomic analysis

To establish if cheese isolates were affiliated with one of the closely related species previously described or are probable new species of *Halomonas*, average nucleotide identity (gANI) analyses with whole-genome sequences were performed. The genome of 12 strains were sequenced and annotated as described below. In order to enable a whole-genome-based reconstruction of its phylogeny, genomes of two *Halomonas* type strains (*H. alkaliphila* DSM 16354^T and *H. venusta* DSM 4743^T) not available so far, were also sequenced and annotated. DNA sequencing was carried out on an Illumina HiSeq device at GATC-Biotech (Konstanz, Germany) in order to generate at least 5 million paired-end reads per genome (150 bases in length). For each strain, *de novo* assembly was performed using SPAdes version 3.9 [17]. Only contigs with length >300 bp and coverage >100 were considered for further study. Annotations were performed using the RAST (Rapid Annotation using Subsystem Technology) server [18]. Basic features of these new whole-genome sequences are presented in Table 1.

The whole genomes of all *Halomonas* species available in the NCBI database were collected, and information on these genomes such as their origin of isolation, numbers of Bioproject and BioSample are shown in Table S4. The sequences of these genomes were aligned with those of the strains obtained in this study and with that of *Carnimonas nigrificans* ATCC BAA-78^T, as an outgroup, using Progressive Mauve software (version 2.4.0) with the default settings. The phylogenomic tree obtained was visualized with Interactive Tree of Life (iTOL v5) (<http://itol.embl.de>). Finally, the gANI values of the *Halomonas* strains isolated here and that of closely related species were calculated using JSpeciesWS [19] in order to confirm speciation of the different isolates. For species delineation, we used the recommended cut-off point of 95-96% ANI, which replaces the DDH analysis [20].

Table 1. Genomic features of *Halomonas alkaliphila* and *Halomonas venusta* type strains and the 12 strains representative of the novel *Halomonas* species proposed.

Species	Strain	No of contigs	Size (base pairs)	GC content (%)	N50 ^a	L50 ^b	Number of subsystems (RAST)	Number of coding sequences	Bioproject	BioSample	Accession
<i>Halomonas citridevorans</i>	FME63 ^T	77	3,927,099	54.2	131,964	6	358	3,767	PRJNA501839	SAMN15150959	JABUYX000000000
<i>Halomonas citridevorans</i>	1M45	78	3,759,973	54.2	116,969	10	339	3,641	PRJNA501839	SAMN15150965	JABUZY000000000
<i>Halomonas citridevorans</i>	FME16	62	3,710,911	53.9	157,431	7	364	3,606	PRJNA501839	SAMN10346435	RRZC000000000
<i>Halomonas citridevorans</i>	JB380	65	3,978,282	53.8	160,683	7	476	3,788	PRJEB19045	SAMEA80403418	ERS1545319
<i>Halomonas minus</i>	FME64 ^T	48	3,871,780	53.8	236,760	7	362	3,695	PRJNA501839	SAMN15150960	JABUYZ000000000
<i>Halomonas minus</i>	FME1	57	3,858,166	53.8	227,665	7	352	3,696	PRJNA501839	SAMN10346434	RRZD000000000
<i>Halomonas casei</i>	FME65 ^T	143	4,336,586	53	86,850	19	366	4,168	PRJNA501839	SAMN15150961	JABUYZ000000000
<i>Halomonas casei</i>	3F2F	197	4,339,107	53	NA*	NA*	455	4,081	PRJNA501839	SAMN15150967	JABUZE000000000
<i>Halomonas casei</i>	3D7M	278	4,231,009	52.9	NA*	NA*	426	3,916	PRJNA501839	SAMN15150968	JABUZF000000000
<i>Halomonas casei</i>	3A7M	175	4,120,177	52.9	56,190	22	340	3,992	PRJNA501839	SAMN15150966	JABUZD000000000
<i>Halomonas flavum</i>	FME66 ^T	122	4,506,877	54.2	76,473	18	379	4,365	PRJNA501839	SAMN15150962	JABUZA000000000
<i>Halomonas flavum</i>	FME20	194	4,221,656	54.2	67,999	20	366	4,065	PRJNA501839	SAMN10346436	RRZB000000000
<i>Halomonas alkaliphila</i>	DSM 16354 ^T	45	4,095,328	52.6	321,391	5	357	3,909	PRJNA625506	SAMN14601836	JABASV000000000
<i>Halomonas venusta</i>	DSM 4743 ^T	59	4,307,261	52.7	313,561	6	355	4,089	PRJNA625506	SAMN14601835	JABASW000000000

Figure 3 presents a phylogenomic tree obtained with *Halomonas* whole-genome sequences with the indication of their environmental origins such as aquatic environments (sea, salt lakes), terrestrial environments (metal sulfide rock and soil) as well as food (Table S4). Whole-genome ANI values of cheese strains and phylogenetically related species showed that the cheese strains were clustered in four different genomic clades probably affiliated with four new species (Fig. 4). The four groups of cheese strains represented by FME63^T, FME64^T, FME65^T and FME66^T shared 82.4, 79.9, 95.3 and 93.1% ANI with the relative closest type strains of *H. zhanjiangensis* (JSM 078169^T), *H. boliviensis* (LC1^T), *H. alkaliphila* (DSM 16354^T) and *H. zhanjiangensis* (JSM 078169^T), respectively.

In the group of the four strains represented by FME63^T, strains JB380 and FME16 display ANI values at the limit of definition of a new species ($\approx 95\%$) with FME63^T, and splitting or merging to a species is subject to interpretation by taxonomists [21]. In the present case, physiological analyses will be essential to definitively determine their status.

Additionally, the group of four strains represented by FME65^T share ANI of 93.5% with *H. venusta* DSM 4743^T and 95.3% with *H. alkaliphila* DSM 16354^T (Fig. 4). This last value is also in the boundary range (95-96%) of species delimitation. ANI analyses with additional genomes of strains belonging to *H. venusta* and *H. alkaliphila* species showed three distinct homogeneous clades with ANI value $> 98\%$ (Table S5). This data, in agreement with the phylogenomic tree (Fig. 3), indicates that the four cheese isolates form a separate group with the closest related species (*H. alkaliphila* and *H. venusta*), suggesting that each clade could be considered as a different species. Nevertheless, since ANI values are at the boundary range, we consider it essential to supplement these data with physiological analyses to secure the classification of these strains.

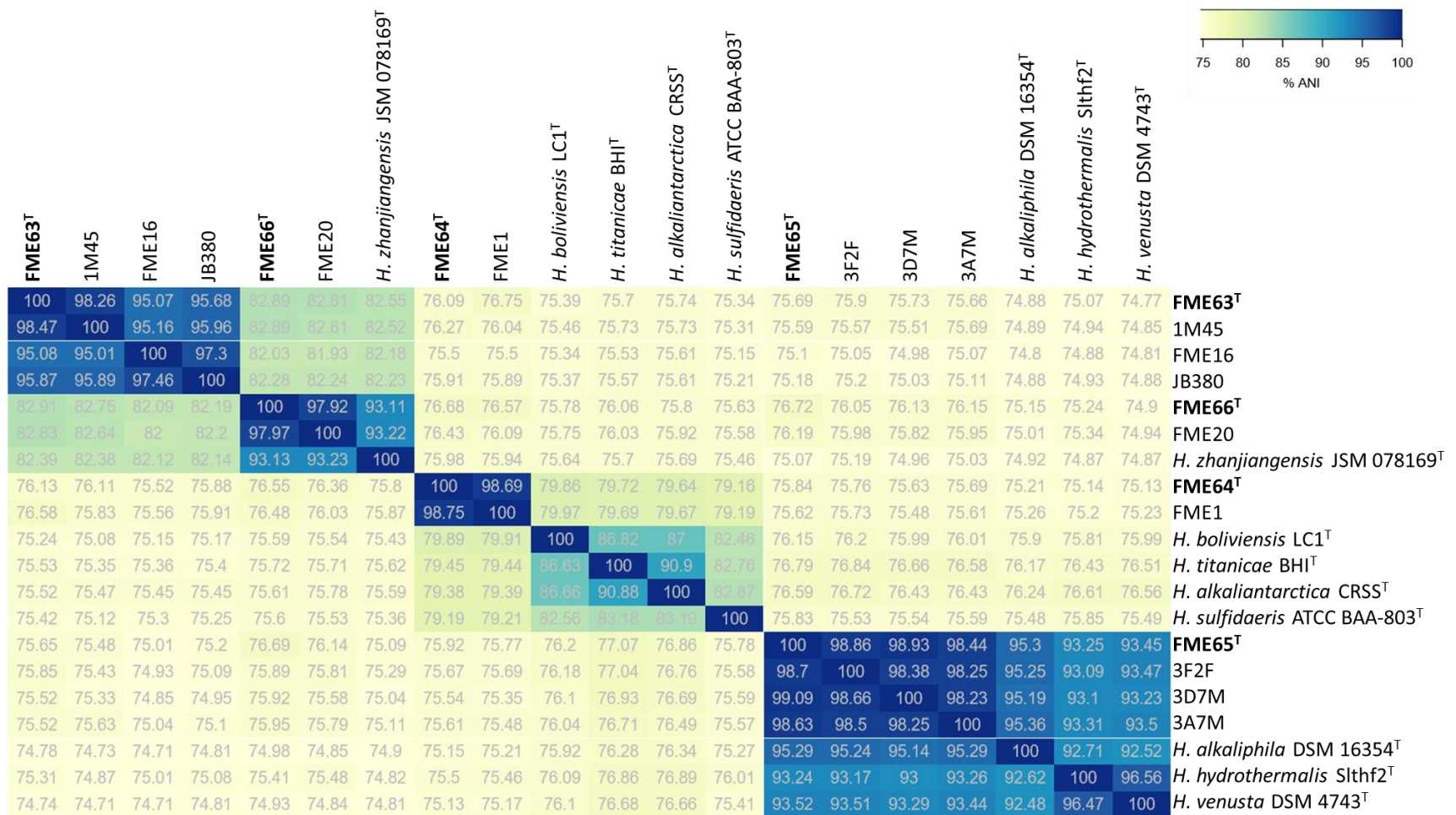


Figure 4. Heatmap depicting the average nucleotide identity (ANI) of the cheese-associated strains investigated in the present study and closely related *Halomonas* species. Type strains from this study are highlighted in bold.

Physiological, enzymatic and biochemical properties

Physiological characteristics, including cell morphology and motility, were observed using an optical microscope after culture for 48 h on Tryptic Soy Agar (TSA) plates. Growth was tested at various temperatures (4, 10, 20, 25, 30 and 37°C), and NaCl concentrations (0, 0.5, 2, 5, 8, 12, 16 and 20%) and pH values (4, 5, 6, 7, 8, 9 and 10) on TSA and TSB after culture for 72 h. Additionally, enzymatic activities were tested with the API ZYM system (bioMérieux, Bruz, France) according to the manufacturer's instructions, except that the strips were incubated for 6 h at 25°C. The API 20NE system (bioMérieux, Bruz, France) was used to test biochemical properties and was prepared according to the manufacturer's instructions.

Distinctive characteristics that differentiate the newly described strains from type strains of closely related phylogenetic species are shown in [Table 2](#), and supplementary characteristics are available in [Table S6](#). All *Halomonas* strains tested are rods or short rods, aerobic, can grow at pH 6-10 and support up to 16% NaCl. Overall, on the basis of the physiological, enzymatic and biochemical data, we noted that the species tested shared a high similarity in terms of these properties ([Table S6](#)). Mata *et al.* (2002) [24] confirmed the difficulty of distinguishing the species of the genus *Halomonas* by its phenotypic characteristics. However, we found several properties that are critical to discriminate the different species, in combination with the genomic analyses previously described.

The group consisting of the strains FME63^T, 1M45, FME16 and JB380 belongs to a new species because it presents a significant distance from any defined species (closest ANI ≈82% with *H. zhanjiangensis*). The lowest value of ANI within this group of 95% ([Fig. 4](#)) suggests the presence of two different genomic clusters. Nevertheless, physiological analyses showed high similarity between these four strains, crucial for defining them as a single species. Additionally, this group contains the only cheese strains capable of assimilating trisodium citrate and differs from *H. zhanjiangensis* by the non-assimilation of potassium gluconate and by the cream colour of their colonies ([Table 2](#)).

Table 2. Distinctive physiological, enzymatic and biochemical properties that differentiate the novel *Halomonas* strains from closely related species of the genus

Halomonas.

Characteristics	FME63 ^T	1M45	FME16	JB380	FME66 ^T	FME20	<i>H.</i> <i>zhanjiangensis</i> JSM 078169 ^T	FME64 ^T	FME1	<i>H.</i> <i>boliviensis</i> LC1 ^T *	FME65 ^T	3F2F	3D7M	3A7M	<i>H.</i> <i>alkaliphila</i> DSM 16354 ^T	<i>H.</i> <i>venusta</i> DSM 4743 ^T
Colony color	cream	cream	cream	cream	yellow	yellow	yellow	cream/ white	cream/ white	cream	cream	cream	cream	cream	cream	cream
Colony diameter (mm)	2	2.5	3	3	3	2	3	1.5	2	ND	2.5	2.5	3	2	3	3
Oxidase	-	-	-	-	-	-	-	-	-	ND	+	+	+	+	+	+
Esterase	+	+	+	-	+	+	+	+	+	ND	+	+	+	+	-	-
Valine arylamidase	+	-	+	+	-	+	-	-	-	ND	+	+	+	+	+	+
α -glucosidase	-	-	-	-	-	-	-	-	-	ND	+	+	+	+	+	+
Nitrate reduction	-	+	+	+	-	-	-	-	-	+	-	+	+	-	+	+
Mannitol assimilation	-	-	-	-	-	-	-	-	-	ND	-	-	-	-	-	+
N-acetyl- glucosamine assimilation	-	-	-	-	-	-	-	-	-	ND	-	-	-	-	+	+
Maltose assimilation	-	-	-	-	-	-	-	-	-	ND	-	-	-	-	+	+
Potassium gluconate assimilation	-	-	-	-	+	+	+	-	-	ND	-	-	-	-	+	-
Adipic acid assimilation	-	-	-	+	-	+	+	-	+	ND	-	-	-	-	+	-
Trisodium citrate assimilation	+	+	+	+	-	-	+	-	-	ND	-	-	-	-	+	-

* Data from Quillaguamán *et al.* (2004)

ND: Not determined

The FME64^T and FME1 strains belong to a group that is taxonomically distant from those in the database (ANI < 80% with the closest outside species), clearly indicating their status as a new species. Additionally, their colonies present relatively smaller diameters in comparison with the other evaluated species, and nitrate reduction is a reaction that distinguishes this group from the closest species (*H. boliviensis* LC1^T).

The group of strains represented by FME65^T, 3F2F, 3D7M and 3A7M shares ANI values in the boundary zone of the species delimitation (95-96%) with *H. alkaliphila* DSM 16354^T, raising doubts about their assignment to this species. Nevertheless, the combination of ANI (Fig. 4 and Table S5), and phylogenomic analyses showed that the isolates of this study belong to a distinct group (Fig. 3). Moreover, the cheese strains exhibit a combination of reactions that discriminate them from *H. alkaliphila*, including esterase positive test and non-assimilation of N-acetyl-glucosamine, maltose, potassium gluconate, adipic acid and trisodium citrate (Table 2). These results confirm the fact that they effectively correspond to a distinct species.

Finally, the FME66^T and FME20 cheese strains can be discriminated from the closest species (*H. zhanjiangensis*) by ANI analysis (<95%, Fig. 4). These strains are distinguished by the yellow pigmentation of their colonies, while the other cheese strains are white or cream-colored. Moreover, the non-assimilation of trisodium citrate differentiates these strains from the closest related species *H. zhanjiangensis*, indicating that they can be assigned to a new species.

Therefore, based on the results presented here, these 12 cheese strains are considered as being representative of four new *Halomonas* species, for which the names *Halomonas citridevorans* sp. nov. (strains FME63^T, 1M45, FME16 and JB380), *Halomonas minus* sp. nov. (strains FME64^T and FME1), *Halomonas casei* sp. nov. (strains FME65^T, 3F2F, 3D7M and 3A7M) and *Halomonas flavum* sp. nov. (strains FME66^T and FME20) are proposed.

Description of *Halomonas citridevorans* sp. nov.

Halomonas citridevorans sp. nov (ci.tri.de.vo'rans. L. n. *citrus*, lemon tree; L. n. *acidunz*, acid; L. neut. n. *acidum citri*, citric acid; L. part. adj. *devorans*, consuming, devouring; referring to trisodium citrate assimilation).

Cells are Gram-negative, motile, aerobic, catalase-positive and oxidase-negative. Colonies are cream-colored, non-translucent and raised with entire margin. Colonies grown on TSA medium at 25°C for 72 h have a diameter of 2-3 mm. Strain growth occurs from 4 to 30°C, but not at 37°C, in a pH range of 6.0 to 10.0, and in the presence of 0.5 to 16% (w/v) NaCl, but not at 0 and 20%. This species is positive for leucine arylamidase and assimilation of trisodium citrate.

The type strain FME63^T was isolated from semi-soft cow's milk cheese in France.

Description of *Halomonas minus* sp. nov.

Halomonas minus sp. nov (mi'nus. L. neut. adj. *minus* smaller, referring to the colony size).

Cells are Gram-negative, aerobic and oxidase-negative. Colonies are cream-colored/white, circular, non-translucent and raised with entire margin. Colonies grown on TSA medium at 25°C for 72 h have a diameter of 1.5-2 mm. Strain growth occurs from 4 to 25°C, but not at 37°C, in a pH range of 6.0 to 10.0, and in the presence of 0.5 to 16% (w/v) NaCl, but not at 0 and 20%. This species is positive for esterase, esterase lipase, leucine arylamidase and Naphthol AS-BI-phosphate.

The type strain FME64^T was isolated from semi-soft cow's milk cheese in France.

Description of *Halomonas casei* sp. nov.

Halomonas casei sp. nov (ca.se'i. L. gen. n. *casei* of cheese, referring to origin of isolation).

Cells are Gram-negative, aerobic, catalase- and oxidase-positive. Colonies are beige, circular, non-translucent and raised with entire margin. Colonies grown on TSA medium at 25°C for 72 h have a diameter of 2-3 mm. Strain growth occurs from 10 to 30°C, in a pH range of 6.0

to 10.0, and in the presence of 0.5 to 16% (w/v) NaCl, but not at 0 and 20%. This species is positive for esterase, leucine arylamidase and α -glucosidase.

The type strain FME65^T was isolated from semi-soft cow's milk cheese in France.

Description of *Halomonas flavum* sp. nov.

Halomonas flavum (fla'vum. L. neut. adj. *flavum* yellow, referring to the colony color).

Cells are Gram-negative, aerobic, catalase-positive and oxidase-negative. Colonies are yellow pigmented, circular, non-translucent and raised with entire margin. Colonies grown on TSA medium at 25°C for 72 h have a diameter of 2-3 mm. Strain growth occurs from 4 to 30°C, but not at 37°C, in a pH range of 6.0 to 10.0, and in the presence of 0.5 to 16% (w/v) NaCl, but not at 20%. This species is positive for esterase, esterase lipase, leucine arylamidase and Naphthol AS-BI-phosphate, and was able to assimilate potassium gluconate and malate.

The type strain FME66^T was isolated from semi-soft cow's milk cheese in France.

AUTHOR STATEMENTS

Authors and contributors

Caroline Isabel Kothe: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing-original draft, Writing-review & editing.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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ABBREVIATIONS

ANI: Average Nucleotide Identity

TSA: Tryptic Soy Agar

TSB: Tryptic Soy Broth

SUPPLEMENTARY MATERIAL

The supplementary Tables S1-S6 can be found at <http://dx.doi.org/10.17632/7vc3vndc3r.1>.

Table S1. Origin, media and temperatures of isolation of the new *Halomonas* strains.

Species	Strain	Source	Media	Temperature (°C)
<i>Halomonas citridevorans</i>	FME63 ^T	Semi-soft cow's milk cheese	MB	25
<i>Halomonas citridevorans</i>	1M45	Soft smear-ripened cheese with pasteurized milk	BHI	28
<i>Halomonas citridevorans</i>	FME16	Soft ewe's milk cheese with natural rind	HM	25
<i>Halomonas citridevorans</i>	JB380	Cheese rind	NA	NA
<i>Halomonas minus</i>	FME64 ^T	Semi-soft cow's milk cheese	MB	25
<i>Halomonas minus</i>	FME1	Semi-soft cow's milk cheese	MB	25
<i>Halomonas casei</i>	FME65 ^T	Semi-soft cow's milk cheese	MB	25
<i>Halomonas casei</i>	3F2F	Soft smear-ripened cheese with pasteurized milk	BHI	28
<i>Halomonas casei</i>	3D7M	Soft smear-ripened cheese with pasteurized milk	BHI	28
<i>Halomonas casei</i>	3A7M	Soft smear-ripened cheese with pasteurized milk	BHI	28
<i>Halomonas flavum</i>	FME66 ^T	Semi-soft cow's milk cheese	MB	25
<i>Halomonas flavum</i>	FME20	Semi-soft cow's milk cheese	MB + 4% NaCl	25

MB = Marine Broth 2216 (BD Difco, Sparks, MD); HM = Halomonas medium*; BHI = Brain Heart Infusion (BD Difco, Sparks, MD); NA = Not Available.

*Vreeland RH, Martin EL. Growth characteristics, effects of temperature, and ion specificity of the halotolerant bacterium *Halomonas elongate*. Can J Microbiol 1980; 26:746–752.

Table S2. Accession numbers of 16S rRNA gene sequences used to build the phylogenetic tree shown in Fig. 1.

Table S3. Identities of 16S rRNA and *rpoB* gene sequences (%) between the cheese-associated strains investigated in the present study and closely related species. Identity values are highlighted in green when species delineation is above the threshold (>99% for 16S rRNA and >97.7% for *rpoB* gene sequences). **(A)** *H. citridevorans*; **(B)** *H. minus*; **(C)** *H. casei*; **(D)** *H. flavum*.

(A) *H. citridevorans*

Species and/or Strains	16S rRNA gene				<i>rpoB</i> gene			
	FME63 ^T	1M45	JB380	FME16	FME63 ^T	1M45	JB380	FME16
<i>H. citridevorans</i> FME63 ^T		99.91	99.48	99.22		100	97.25	97.38
1M45	99.91		99.26	99.08	100		97.25	99.63
JB380	99.48	99.26		99.48	97.25	97.25		97.38
FME16	99.22	99.08	99.48		97.38	97.38	99.63	
<i>H. nigrificans</i> MBT G8648 ^T	98.15	98.16	98.35	98.21	88.26	88.26	88.11	88.21
<i>H. titanicae</i> BH1 ^T	98.08	98.25	97.73	97.53	88.41	88.53	88.02	88.12
<i>H. alkaliantarctica</i> CRSS ^T	97.46	97.70	97.73	98.08	88.53	88.53	88.06	88.13
<i>H. boliviensis</i> LC1 ^T	97.32	97.52	97.59	97.94	87.62	87.62	87.67	87.16
<i>H. olivaria</i> C17 ^T	97.19	97.43	97.32	97.66	88.25	88.25	87.67	87.80
<i>H. sulfidaeris</i> ATCC BAA-803 ^T	96.87	97.50	96.87	97.28	87.49	87.49	87.32	87.49
<i>H. glacei</i> DD 39 ^T	98.08	98.53	97.73	98.08				
<i>H. utahensis</i> DSM 3051 ^T	97.94	98.44	97.59	97.94				
<i>H. neptunia</i> Eplume1 ^T	97.32	97.52	97.46	97.80				

(B) *H. minus*

Species and/or Strains	16S rRNA gene		<i>rpoB</i> gene	
	FME64 ^T	FME1	FME64 ^T	FME1
<i>H. minus</i> FME64 ^T		100		98.14
FME1	100		98.14	
<i>H. alkaliantarctica</i> CRSS ^T	98.76	98.76	90.75	90.73
<i>H. olivaria</i> C17 ^T	98.49	98.49	90.11	90.18
<i>H. boliviensis</i> LC1 ^T	98.56	98.56	89.85	89.92

(C) *H. casei*

Species and/or Strains	16S rRNA gene				<i>rpoB</i> gene			
	FME65 ^T	3F2F	3D7M	3A7M	FME65 ^T	3F2F	3D7M	3A7M
<i>H. casei</i> FME65^T		100	100	99.71		100	100	100
3F2F	100		100	99.81	100		100	100
3D7M	100	100		99.71	100	100		100
3A7M	99.71	99.81	99.71		100	100	100	
<i>H. alkaliphila</i> DSM 16354 ^T	99.86	99.81	99.86	99.64	98.36	98.36	98.36	98.36
<i>H. venusta</i> DSM 4743 ^T	99.52	99.52	99.52	99.5	96.15	96.15	96.15	96.15
<i>H. hydrothermalis</i> Slthf2 ^T	99.57	99.62	99.57	99.28	94.53	94.53	94.53	94.53
<i>H. zhaodongensis</i> NEAU-ST10-25 ^T	99.35	99.33	99.35	99.27				

(D) *H. flavum*

Species and/or Strains	16S rRNA gene		<i>rpoB</i> gene	
	FME66 ^T	FME20	FME66 ^T	FME20
<i>H. flavum</i> FME66^T		100		99.68
FME20	100		99.68	
<i>H. zhanjiangensis</i> JSM 078169 ^T	99.78	99.52	98.68	98.55
<i>H. nanhaiensis</i> YIM M 1305 ^T	98.57	98.63		

Table S4. Information concerning *Halomonas* strains whose whole-genome sequences were used to build the phylogenomic tree in Fig. 3.

Species	Strain	Type strain	Origin	Country
<i>Halomonas aestuarii</i>	Hb3	Yes	Tidal flat	South Korea
<i>Halomonas alimentaria</i>	DSM 15356	Yes	Fermented food	South Korea
<i>Halomonas alkaliantarctica</i>	CRSS	Yes	Saline lake	Ross Sea
<i>Halomonas alkaliphila</i>	DSM 16354	Yes	Salt pool	Italy
<i>Halomonas andesensis</i>	DSM 19434	Yes	Saline lake water	Bolivia
<i>Halomonas anticariensis</i>	DSM 16096	Yes	Soil from a saline wetland	Spain
<i>Halomonas aquamarina</i>	558	Yes	Seawater	Unknown
<i>Halomonas arcis</i>	CGMCC 1.6494	Yes	Salt lake	China
<i>Halomonas axialensis</i>	Althf1	Yes	Sulfide rock	Pacific Ocean
<i>Halomonas beimenensis</i>	NTU-111	No	Unkown	Taiwan
<i>Halomonas boliviensis</i>	LC1	Yes	Soil	Bolivia
<i>Halomonas borealis</i>	ATF 5.2	Yes	Sea water	Norway
<i>Halomonas caseinilytica</i>	JCM 14802	Yes	Soil	China
<i>Halomonas chromatireducens</i>	AGD 8-3	No	Soil	Russia
<i>Halomonas coralii</i>	362.1	Yes	Coral	Brazil
<i>Halomonas cupida</i>	DSM 4740	Yes	Seawater	USA
<i>Halomonas daqiaoogenesis</i>	CGMCC 1.9150	Yes	Littoral saltern	China
<i>Halomonas daqingensis</i>	CGMCC 1.6443	Yes	Oilfield soil	China
<i>Halomonas denitrificans</i>	DSM 18045	Yes	Saline water	South Korea
<i>Halomonas desiderata</i>	FB2	Yes	Sewage treatment plant	Germany
<i>Halomonas elongata</i>	DSM 2581	Yes	Solar salt facility	Netherlands Antilles
<i>Halomonas endophytica</i>	MC28	Yes	Storage liquid in the stem	China
<i>Halomonas eurihalina</i>	MS1	No	Saline soils	Spain
<i>Halomonas gudaonensis</i>	CGMCC 1.6133	Yes	Saline soil contaminated	China
<i>Halomonas halmophila</i>	NBRC 15537	No	Unkown	Unkown
<i>Halomonas halocynthiae</i>	DSM 14573	Yes	Sea peach	Japan
<i>Halomonas halodenitrificans</i>	DSM 735	Yes	Meat	Canada
<i>Halomonas halophila</i>	NBRC 102604	No	Hypersaline soil	Spain
<i>Halomonas heilongjiangensis</i>	DSM 26881	Yes	Soil	China
<i>Halomonas huangheensis</i>	BJGMM-B45	Yes	Soil	China
<i>Halomonas hydrothermalis</i>	Slthf2	Yes	Deep-sea hydrothermal vent	Pacific Ocean
<i>Halomonas ilicicola</i>	DSM 19980	Yes	Saline water from a solar saltern	Spain
<i>Halomonas jeotgali</i>	Hwa	Yes	Fermented seafood	South Korea
<i>Halomonas korlensis</i>	CGMCC 1.6981	Yes	Saline and alkaline soil	China
<i>Halomonas lionensis</i>	RHS90	Yes	Sea sediments	Mediterranean Sea
<i>Halomonas litopenaei</i>	SYSU ZJ2214	Yes	Shrimp water	China
<i>Halomonas lutea</i>	DSM 23508	Yes	Salt lake	China
<i>Halomonas massiliensis</i>	Marseille-P2426	Yes	Human gut	France
<i>Halomonas meridiana</i>	ACAM 246	Yes	Organic lake	Antarctica
<i>Halomonas muralis</i>	DSM 14789	Yes	Mural painting	Austria
<i>Halomonas nigrificans</i>	MBT G8648	Yes	Cheese	Germany
<i>Halomonas niordiana</i>	ATF 5.4	Yes	Seawater	Norway

<i>Halomonas nitroreducens</i>	11S	Yes	Solar saltern	Chile
<i>Halomonas olivaria</i>	C17	Yes	Salted olive-processing effluents	Morocco
<i>Halomonas pacifica</i>	NBRC 102220	No	Seawater	USA
<i>Halomonas pantelietiensis</i>	AAP	Yes	Hard sand of the lake of Venere	Italy
<i>Halomonas saccharevitans</i>	CGMCC 1.6493	Yes	Water sample	China
<i>Halomonas salina</i>	B6	No	Human gut	France
<i>Halomonas saliphila</i>	LCB169	Yes	Saline soil	China
<i>Halomonas shengliensis</i>	GCMCC 1.6444	Yes	Contaminated saline soil	China
<i>Halomonas smyrnensis</i>	AAD6	Yes	Soil from salt production ponds	Turkey
<i>Halomonas socia</i>	CKY01	No	Marine soil	South Korea
<i>Halomonas songnenensis</i>	CGMCC 1.12152	Yes	Saline and alkaline soils	China
<i>Halomonas stevensii</i>	S18214	Yes	Blood from a renal care patient	USA
<i>Halomonas subglaciescola</i>	ACAM 12	Yes	Organic lake	Antarctica
<i>Halomonas subterranea</i>	CGMCC 1.6495	Yes	Hypersaline water	China
<i>Halomonas sulfidaeris</i>	ATCC BAA-803	Yes	Metal sulfide rock	Northeast Pacific Ocean
<i>Halomonas taeanensis</i>	BH539	Yes	Solar saltern	Korea
<i>Halomonas titanicae</i>	BH1	Yes	Rusticles of the RMS Titanic wreck	Canada
<i>Halomonas urmiana</i>	TBZ3	Yes	Lake	Iran
<i>Halomonas urumqiensis</i>	BZ-SZ-XJ27	Yes	Sediment from saline-alkaline lake	China
<i>Halomonas utahensis</i>	P-halo	No	Human gut	Unknown
<i>Halomonas ventosae</i>	CECT 5797	Yes	Soil with high salt content	Spain
<i>Halomonas venusta</i>	DSM 4743	Yes	Seawater	Unknown
<i>Halomonas xianhensis</i>	CGMCC 1.6848	Yes	Soil	Unknown
<i>Halomonas xinjiangensis</i>	TRM 0175	Yes	Salt lake	China
<i>Halomonas zhanjiangensis</i>	JSM 078169	Yes	Sea urchin	China
<i>Halomonas zincidurans</i>	B6	Yes	Marine sediment	South Atlantic
Novel cheese strains from this study				
<i>Halomonas citridevorans</i>	FME63	Yes	Semi-soft cow's milk cheese	France
<i>Halomonas citridevorans</i>	1M45	No	Soft smear-ripened cheese with pasteurized milk	France
<i>Halomonas citridevorans</i>	FME16	No	Soft ewe's milk cheese with natural rind	France
<i>Halomonas citridevorans</i>	JB380	No	Cheese rind	USA
<i>Halomonas minus</i>	FME64	Yes	Semi-soft cow's milk cheese	France
<i>Halomonas minus</i>	FME1	No	Semi-soft cow's milk cheese	France
<i>Halomonas casei</i>	FME65	Yes	Semi-soft cow's milk cheese	France
<i>Halomonas casei</i>	3F2F	No	Soft smear-ripened cheese with pasteurized milk	France
<i>Halomonas casei</i>	3D7M	No	Soft smear-ripened cheese with pasteurized milk	France
<i>Halomonas casei</i>	3A7M	No	Soft smear-ripened cheese with pasteurized milk	France
<i>Halomonas flavum</i>	FME66	Yes	Semi-soft cow's milk cheese	France
<i>Halomonas flavum</i>	FME20	No	Semi-soft cow's milk cheese	France
Outgroup				
<i>Carnimonas nigrificans</i>	ATCC BAA-78	Yes	Cured meat product	Unknown

Table S5. ANIb values (%) between the strains of the FME65^T- group and the strains of the *Halomonas alkaliphila-venusta* group. The ANI values are highlighted in green when above 96% and in yellow when ranging between 95 and 96%.

	<i>H. venusta</i> NBRC 102221	<i>H. venusta</i> MA- ZP17-13	<i>H. venusta</i> DSM 4743 ^T	<i>H. alkaliphila</i> DSM 16354 ^T	<i>H. alkaliphila</i> X3	FME65 ^T	3F2F	3D7M	3A7M
<i>H. venusta</i> NBRC 102221	*	98.68	99.99	92.58	92.57	93.46	93.5	93.25	93.4
<i>H. venusta</i> MA-ZP17-13	98.58	*	98.58	92.63	92.58	93.43	93.45	93.17	93.32
<i>H. venusta</i> DSM 4743 ^T	99.9	98.54	*	92.48	92.55	93.52	93.51	93.29	93.44
<i>H. alkaliphila</i> DSM 16354 ^T	92.53	92.68	92.52	*	98.02	95.29	95.24	95.14	95.29
<i>H. alkaliphila</i> X3	92.59	92.62	92.62	98.01	*	95.53	95.48	95.39	95.5
FME65 ^T	93.45	93.37	93.45	95.3	95.4	*	98.86	98.93	98.44
3F2F	93.46	93.39	93.47	95.25	95.33	98.7	*	98.38	98.25
3D7M	93.24	93.18	93.23	95.19	95.28	99.09	98.66	*	98.23
3A7M	93.5	93.45	93.5	95.36	95.46	98.63	98.5	98.25	*

95>ANI>96%	
ANI>96%	

Table S6. Physiological, enzymatic and biochemical characteristics of the cheese-associated strains investigated in the present study and of closely related species.

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3.3. Publication 4

***Psychrobacter casei* sp. nov. and *Psychrobacter translucens* sp. nov., two new species isolated from French cheese rinds**

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Keyword: Taxonomy, sequence analysis, whole-genome sequencing, food.

Repositories:

The whole-genome and 16S sequences of four novel strains of *Psychrobacter* have been deposited at DDBJ/ENA/GenBank and are available under the accession numbers listed in [Table 1](#) and [Figure 1](#), respectively.

ABSTRACT

Four Gram-negative bacterial strains were isolated from cheese rinds in France. On the basis of 16S rRNA gene sequence analysis, all isolates were assigned to the genus *Psychrobacter*, and their closest relatives were *Psychrobacter glacei*, *P. fozii* and *P. aquimaris* with similarities between 98.63 to 99.25%. Their taxonomic status were investigated using a polyphasic taxonomic approach comprising 16S rRNA and *rpoB* phylogenies, determination of average nucleotide identity (gANI) and analyses of physiological and biochemical features. Phylogenetics and gANI analyses revealed the existence of two novel and well-supported clusters. The novel isolates differed from the previously described species by ANI <83% and some biochemical and enzymatic properties. In conclusion, the results of phenotypic and phylogenetic analyses indicated that the isolates belonged two novel *Psychrobacter* species, for which the names *Psychrobacter casei* sp. nov. and *Psychrobacter translucens* sp. nov. are

proposed, with isolates FME6^T (=CIRM-BIA2434^T = CIP 111878^T= CIRM-BIA2435^T) and FME13^T (=CIRM-BIA2435^T = CIP 111879^T = CIRM-BIA2435^T) as type strains, respectively.

Psychrobacter's taxonomic status appears to have been under constant review over the past few years. The first proposed species of this genus was *Psychrobacter immobilis* (in 1986), that was added as part of the *Neisseriaceae* family [1]. However, in 1991, Rossau *et al.* demonstrated that *Psychrobacter* and related organisms (as *Moraxella* and *Acinetobacter*) form a separate cluster in this taxonomy, and the *Moraxellaceae* family was proposed [2, 3]. Therefore, *Psychrobacter* belongs nowadays to the family of *Moraxellaceae* within the class of *Gammaproteobacteria*. Species from this genus are aerobic, Gram-negative, oxidase and catalase-positive, halotolerant, psychrophilic or psychrotolerant [2]. They have been mainly isolated from marine and terrestrial environments, including food, soil, seawater, sea ice and air [3, 4].

Here we describe four strains belonging to the *Psychrobacter* genus, which were isolated from French cheese rinds. Since these strains differed by several unusual genetic and phenotypic features from the closely related *Psychrobacter* type strains, we established they belong to two new species of *Psychrobacter*, named *P. casei* sp. nov. and *P. translucens* sp. nov.

Isolation and Ecology

In the course of a project exploring the diversity of bacteria associated with French artisanal cheese rinds, we isolated four strains, which belong to the genus *Psychrobacter*. These strains (FME5, FME6, FME61 and FME13) were isolated from different types of cheeses (the isolation sources are represented in Table 1) on Marine Agar (MA) at 25°C. Samples obtained from pure colonies were stored at -80°C in Marine Broth (MB) medium supplemented with 20% (v/v) glycerol and deposited in culture collection centers.

Table 1. Genomic features and isolation sources of the novel proposed *Psychrobacter* strains and four *Psychrobacter* type strains (*P. cibarius* DSM 16327^T, *P. aquimaris* DSM 16329^T, *P. faecalis* DSM 14664^T and *P. namhaensis* DSM 16330^T).

Species	<i>Psychrobacter casei</i>	<i>Psychrobacter casei</i>	<i>Psychrobacter translucens</i>	<i>Psychrobacter translucens</i>	<i>Psychrobacter aquimaris</i>	<i>Psychrobacter cibarius</i>	<i>Psychrobacter faecalis</i>	<i>Psychrobacter namhaensis</i>
Strain	FME5	FME6 ^T	FME61	FME13 ^T	DSM 16329 ^T	DSM 16327 ^T	DSM 14664 ^T	DSM 16330 ^T
Isolation source	Semi-soft blue cheese rind with unpasteurized milk from Auvergne-Rhone-Alpes region	Soft-ripened goat's milk cheese from Mediterranean region	Soft smear-ripened cheese rind from Bourgogne region	Soft ewe's milk cheese rind from Mediterranean region	Sea water of the South Sea in Korea	Jeotgal, a traditional Korean fermented seafood	Bioaerosol of the Germany	Sea water of the South Sea in Korea
Number of contigs	295	268	452	419	41	78	106	45
Size (base pairs)	3,373,895	3,361,643	3,569,320	3,708,697	3,427,332	3,242,921	3,081,191	2,900,373
GC content (%)	42.8	42.8	41.7	41.8	43.0	42.8	43.6	45
N50 ^a	24,719	30,966	18,000	20,335	199,424	97,459	67,147	180,725
L50 ^b	38	35	55	50	6	7	15	5
Number of subsystems (RAST)	284	277	284	286	279	272	272	275
Number of coding sequences	3,117	3,067	3,396	3,557	3,109	2,879	2,737	2,581
Bioproject	PRJNA501839	PRJNA501839	PRJNA501839	PRJNA501839	PRJNA625506	PRJNA625506	PRJNA625506	PRJNA625506
BioSample	SAMN10346440	SAMN10346441	SAMN15150957	SAMN10346442	SAMN14601840	SAMN14601841	SAMN14601838	SAMN14601839
Accession	RRYX00000000	RRYW00000000	JABUYV00000000	RRYV00000000	JABASR00000000	JABASQ00000000	JABAST00000000	JABASS00000000

16S rRNA and *rpoB* phylogenies

Genomic DNA was extracted from the bacterial cells according to the protocol described by Almeida *et al.* (2014) [5]. Their 16S rRNA genes were amplified using 27-F (5'-AGAGTTTGATCATGGCTCA-3') and 1492-R (5'-TACGGTTACCTTGTTACGACTT-3') primers [6] and sequences were compared with those of type strains of species with validly published names using EzBioCloud (<https://www.ezbiocloud.net/>). In order to expand the taxonomy of strains, *rpoB* (DNA-directed RNA polymerase beta subunit), as alternative marker gene, was amplified using primers VIC4 (5'-GGCGAAATGGCDGARAACCA-3') and VIC6 (5'-GARTCYTCGAAGTGGTAACC-3') [7]. The *rpoB* sequences were compared to known bacterial species in the National Center for Biotechnology Information (NCBI) GenBank database. Further phylogenetic analyses were performed for 16S rRNA and *rpoB* sequences using MEGA 7.0.26 software and trees were generated using the Neighbor-Joining algorithm with 1,000 bootstrap iterations [8] (Fig. 1 and Fig. 2). To delineate species, we used as threshold over than 99% identity for 16S rRNA gene with type strains [9] and above 97.7% identity for *rpoB* nucleotidic sequence [10].

The strains FME5 and FME6^T are closely related to each other, sharing identities of 99.94% for 16S rRNA (Fig. 1) and 100% for *rpoB* gene sequences (Fig. 2). These strains could be representative of a new genomospecies since their level of identity with the closest related type strains is at the limit of the threshold for 16S rRNA (99.0-99.2%) and below the thresholds for *rpoB* gene (89.0-90.6%) sequences (Table S1A).

Similarly, analyses of 16S rRNA and *rpoB* genes indicated that FME13^T and FME61 strains belong to the same species since their sequences share identities of 99.42 and 100%, respectively. However, they represent probably a new species since their *rpoB* sequences display sub-thresholds levels of identity with all defined species (<92%), despite their 16S rRNA genes is at the limit of species delineation (98.63-99.25%) with *P. aquimaris* SW-210^T (Table S1B).

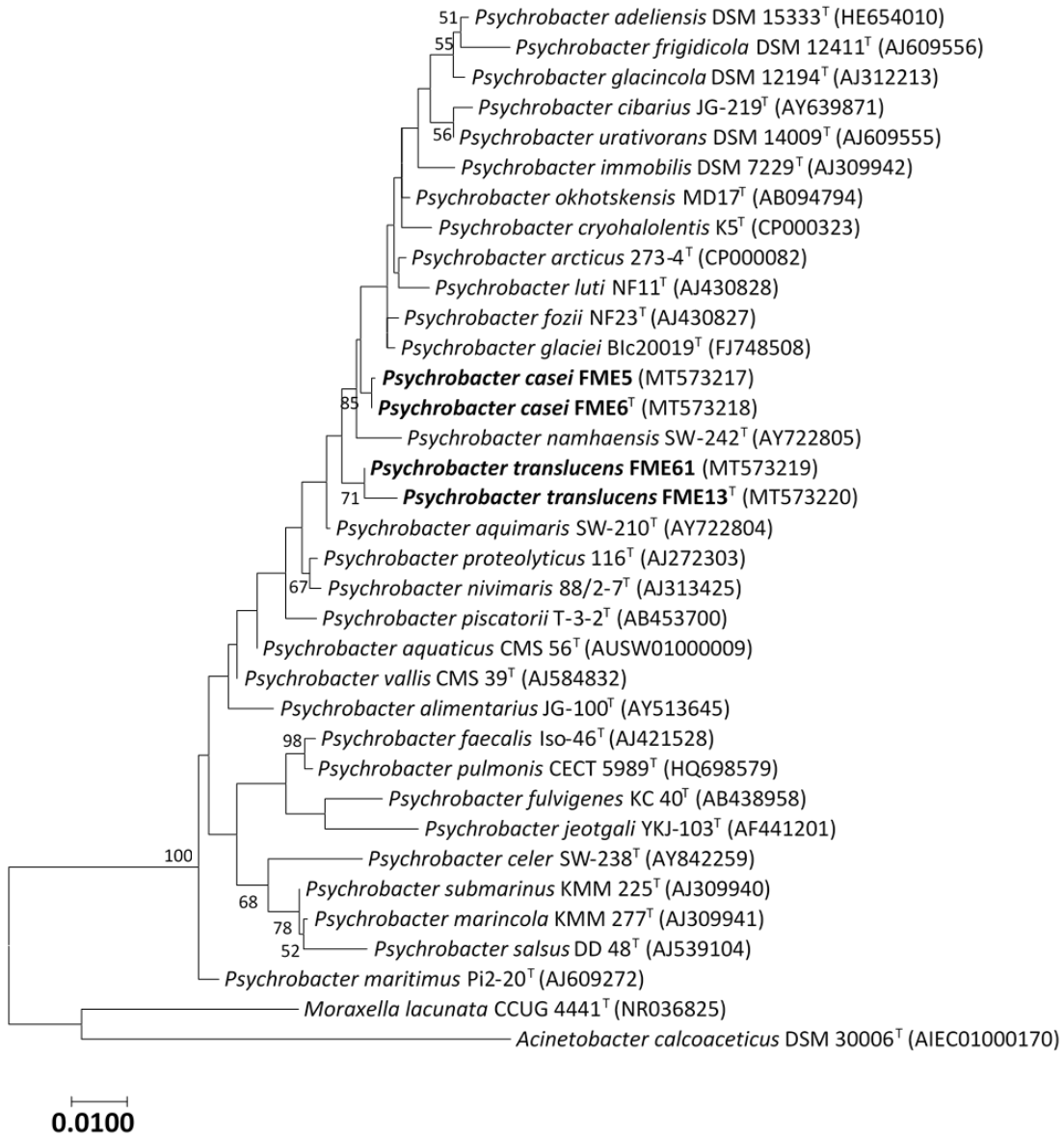


Figure 1. Phylogenetic tree based on 16S rRNA gene sequences of the four strains from this study and closely related *Psychrobacter* type strains. The tree was performed by MEGA 7.0.26 software using the maximum likelihood method. *Acinetobacter calcoaceticus* DSM 30006^T and *Moraxella lacunata* CCUG 4441^T were used as outgroups.

Although the inferred phylogenetic trees confirmed the two well-supported clusters (Fig. 1 and Fig. 2), we performed further investigations to confirm the assignation of these strains.

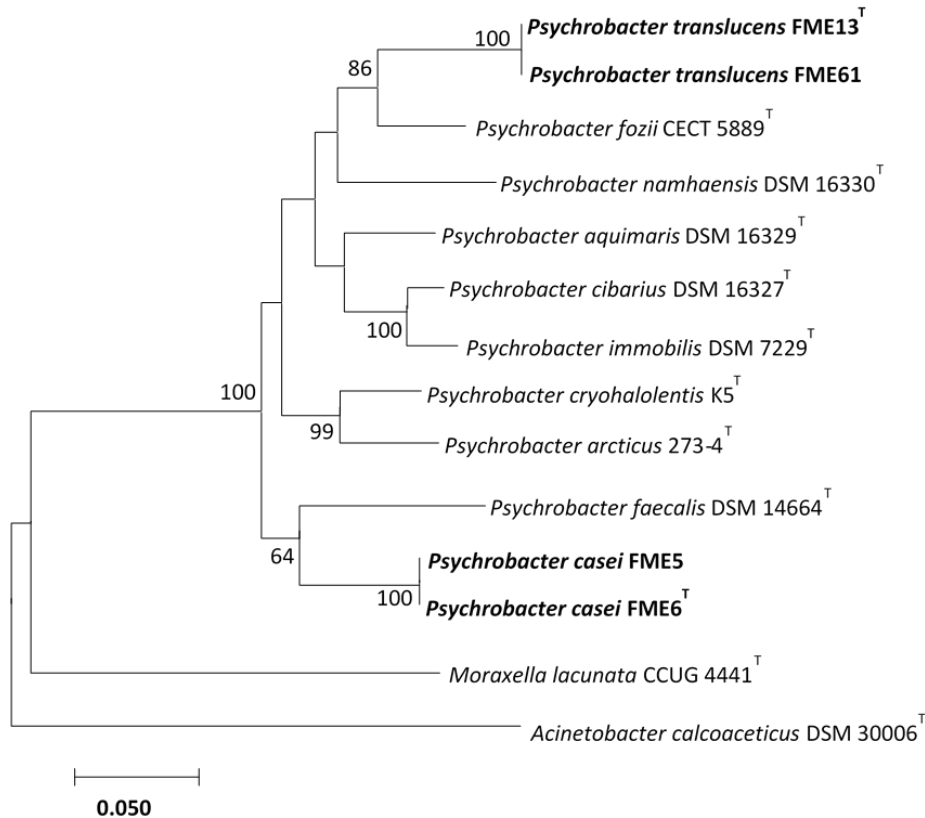


Figure 2. Phylogenetic tree based on *rpoB* gene sequences of the four strains from this study and closely related *Psychrobacter* type strains. The tree was performed by MEGA 7.0.26 software using the maximum likelihood method. *Acinetobacter calcoaceticus* DSM 30006^T and *Moraxella lacunata* CCUG 4441^T were used as outgroups.

Genome features and phylogenomic analysis

To establish if cheese isolates are belonging to new species of *Psychrobacter*, the genome of four strains was sequenced and annotated as described below with the aim of performing an average nucleotide identity (gANI) analyses. Moreover, the genomes of four *Psychrobacter* type strains (*P. cibarius* DSM 16327^T, *P. aquimaris* DSM 16329^T, *P. faecalis* DSM 14664^T and *P. namhaensis* DSM 16330^T), closely related to the cheese strains and which genomes were not available so far, were also determined and annotated in order to complete a whole-genome-based reconstruction of their phylogeny. DNA sequencing was carried out on the Illumina HiSeq platform at GATC-Biotech (Konstanz, Germany) in order to generate at least 5 million paired-end reads per genome (150 bases in length). For each strain, de novo assembly was performed using

SPAdes version 3.9 [11]. Only contigs with length >300 bp and coverage >100 were considered for further study. Annotations were performed using the RAST (Rapid Annotation using Subsystem Technology) server [12]. Basic features of these new whole-genome sequences are presented in Table 1. Finally, these genomes and those of all other *Psychrobacter* species available in the NCBI database were collected and related information such as their origin of isolation, numbers of Bioproject and BioSample are presented in Table S2.

In order to propose a phylogenomic analysis, the available *Psychrobacter* genome sequences were aligned, with those of *Acinetobacter calcoaceticus* DSM 30006^T and *Moraxella lacunata* CCUG 4441^T as outgroups, using the software Progressive Mauve (version 2.4.0) with the default settings. The phylogenomic tree obtained was visualized with Interactive Tree of Life (iTOL v5) (<http://itol.embl.de>). In addition, gANI values of the *Psychrobacter* strains isolated here and that of closely related species were calculated by using JSpeciesWS [13] in order to determine the speciation of the different isolates. For species delineation, we used the recommended cut-off point of 95-96% ANI, which substitutes the DDH analysis [14].

Figure 3 presents the phylogenomic tree obtained with *Psychrobacter* whole-genome sequences with the indication of their origins classified as marine environments, terrestrial environments (sediment, permafrost and soil), human, animal and food (Table S2). Analyses of gANI values of cheese strains and phylogenetically related species showed that the cheese strains are clustered in two different genomic groups of gANI>98% (Table 2). The two groups of cheese strains represented by FME13^T and FME6^T share ≈82.6 and 80.3% ANI with the relative closest type strains of *P. fozii* CECT 5889^T and *P. faecalis* DSM 14664^T (Fig. 3), respectively, a value clearly below the threshold of 95%, indicating their belonging to new species .

Table 2. ANIb values (%) of novel strains and closely related strains of the genus *Psychrobacter*. The ANI values are highlighted in green when species delineation are above of the threshold (>95%).

	FME13 ^T	FME61	<i>P. fozii</i> CECT 5889 ^T	<i>P.</i> <i>namhaensis</i> DSM 16330 ^T	<i>P.</i> <i>aquimaris</i> DSM 16329 ^T	<i>P.</i> <i>cibarius</i> DSM 16327 ^T	<i>P. immobilis</i> DSM 7229 ^T	<i>P.</i> <i>cryohalolentis</i> K5 ^T	<i>P.</i> <i>arcticus</i> 273-4 ^T	<i>P. faecalis</i> DSM 14664 ^T	FME5	FME6 ^T
FME13 ^T	*	98.33	82.67	79.65	80.4	80.67	80.54	78.59	79.23	79.42	80.79	80.84
FME61	98.46	*	82.64	79.93	80.29	80.5	80.56	78.63	79.09	79.69	80.52	80.48
<i>P. fozii</i> CECT 5889 ^T	82.65	82.54	*	79.66	80.67	80.81	80.94	78.44	79.02	78.25	79.33	79.24
<i>P. namhaensis</i> DSM 16330 ^T	79.44	79.53	79.77	*	80.79	80.34	80.58	78.55	78.93	78.82	79.88	79.88
<i>P. aquimaris</i> DSM 16329 ^T	80.26	80.06	80.81	80.68	*	89.68	89.89	79.97	80.21	79.77	80.75	80.71
<i>P. cibarius</i> DSM 16327 ^T	80.27	80.13	80.91	80.3	89.74	*	95.62	79.97	80.32	80.04	80.87	80.96
<i>P. immobilis</i> DSM 7229 ^T	80.21	80.13	80.91	80.59	89.89	95.45	*	80.15	80.39	80.12	80.85	80.93
<i>P. cryohalolentis</i> K5 ^T	78.42	78.37	78.73	78.62	80.19	80.25	80.41	*	88.15	80.19	79.83	79.84
<i>P. arcticus</i> 273-4 ^T	79.07	78.96	79.39	79.23	80.55	80.63	80.67	88.53	*	80.48	80.63	80.68
<i>P. faecalis</i> DSM 14664 ^T	78.63	78.65	78.24	78.95	79.81	80.07	80.22	80.14	80.4	*	80.31	80.34
FME5	80.87	80.29	79.48	79.98	80.89	80.83	80.85	79.87	80.69	80.67	*	98.28
FME6 ^T	80.56	80.09	79.38	79.85	80.85	80.91	80.82	79.93	80.53	80.5	98.23	*

Physiological, enzymatic and biochemical properties

Physiological characteristics including cell morphology and motility were observed using an optical microscope after culture for 48 h on Tryptic Soy Agar (TSA) plates. Growth was tested at various temperatures (4, 10, 20, 25, 30 and 37°C), NaCl concentration (0, 0.5, 2, 5, 8, 12, 16 and 20%) and pH (4, 5, 6, 7, 8, 9 and 10) on TSA after culture for 72 h. Additionally, enzymatic activities were tested with the API ZYM system (bioMérieux, Bruz, France) according to the manufacturer's instructions, except that the strips were incubated for 6h at 25°C. The API 20NE system (bioMérieux, Bruz, France) was used to test biochemical properties and prepared according to the manufacturer's instructions.

The novel and closely related *Psychrobacter* strains are rods or short rods, aerobic, can grow at pH 6-10 and between 10 and 25°C, and support up to 12% NaCl. All these strains are positive (or weakly positive) for esterase (C4), esterase lipase (C8), leucine arylamidase, naphthol-AS-BI-phosphohydrolase and cytochrome oxidase. All strains are negative for the following enzymatic and biochemical properties: trypsin, α -chymotrypsin, α -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, N-acetyl- β -glucosaminidase, α -mannosidas, N₂ production from nitrates, tryptophanase (indole production), fermentation, arginine dihydrolase, protease (gelatin hydrolysis) and β -galactosidase. The strains were also negative for assimilation of d-glucose, d-mannose, d-mannitol, N-acetylglucosamine, d-maltose, potassium gluconate, capric acid, adipic acid and phenylacetic acid. Distinctive characteristics between the newly described strains and type strains representative of closely related phylogenetic species are shown in [Fig. 3](#).

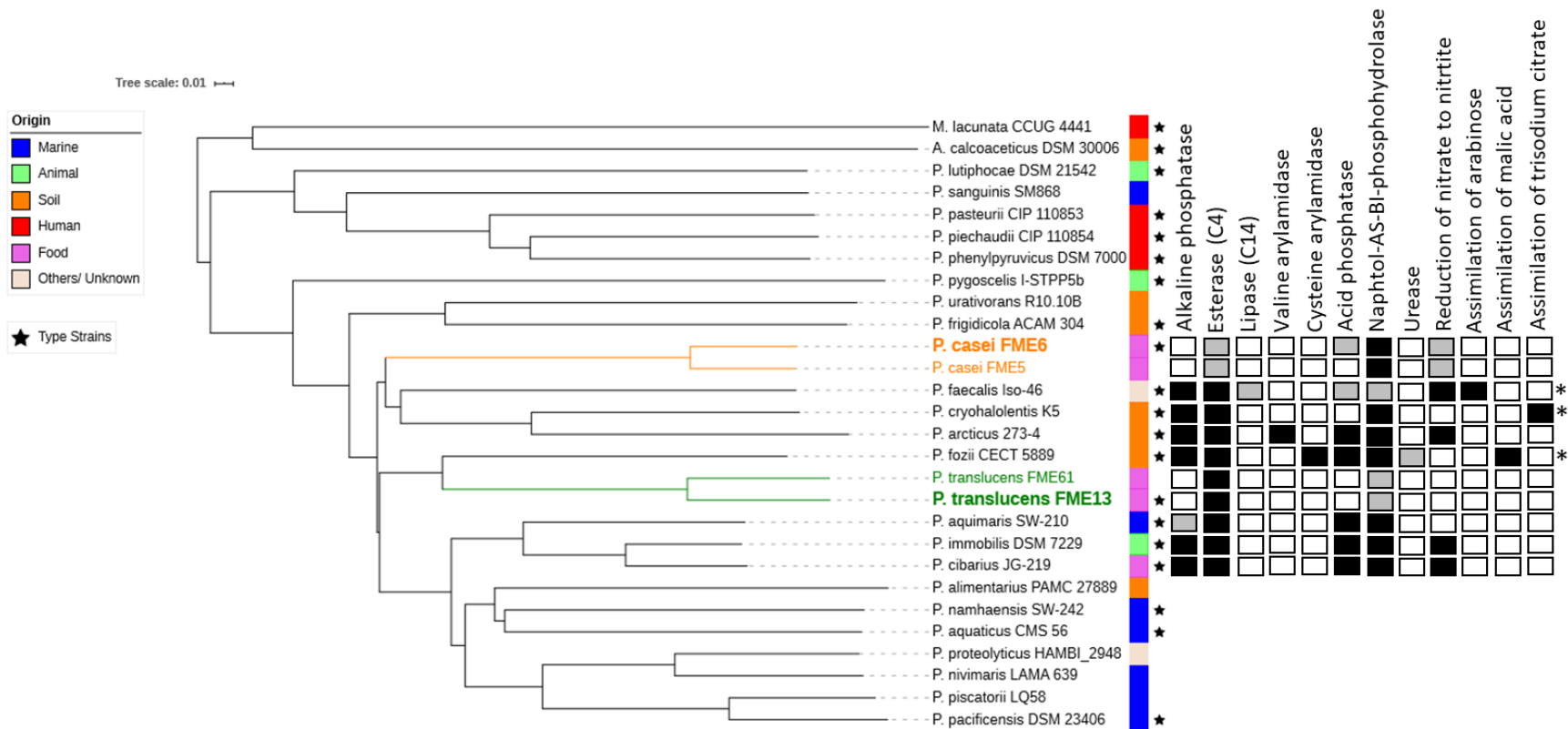


Fig. 3. Phylogenomic tree and biochemical and enzymatic properties. Left: Phylogenomic tree based on whole-genome sequence alignment of all the *Psychrobacter* strains for which this data are available and of the four isolates from this study (highlighted in the tree). Right: Distinctive enzymatic and biochemical properties that differentiate the novel *Psychrobacter* strains from closely related species. Black squares correspond to positive, white squares to negative and grey squares to weakly positive reactions. *Data from *Psychrobacter cryohalolentis*, *P. arcticus* and *P. fozii* were collected from literature (Bakermans *et al.*, 2006 and Bozal *et al.*, 2003).

The group consisting of the strains FME6^T and FME5 belongs to a new species because they present significant distance from any defined species (ANI<81% with the closest outside species). Moreover, these strains are distinguished by the absence of alkaline phosphatase and lipase (C14) and the non-assimilation of arabinose, while in the closest related species (Fig. 3, *P. faecalis*) these reactions are all positive. These results highlight that FME6^T and FME5 strains correspond to a new species.

The FME13^T and FME61 strains were also shown to belong to a taxonomically distant group from those described so far (ANI≈82.6 with *P. fozii*), which clearly indicates they belong to a new species. Additionally, the phenotypic properties that distinguish these two strains from *P. fozii* are the absence of alkaline phosphatase, cysteine arylamidase, acid phosphatase and urease and the non-assimilation of malic acid. Furthermore, the colonies of the FME13^T and FME61 strains are translucent, while those of the closely related species have a cream or beige coloration.

Therefore, based on the results presented here, the four cheese strains are considered as representatives of two new *Psychrobacter* species, for which the names *P. casei* sp. nov. (FME5 and FME6^T strains) and *P. translucens* sp. nov. (FME13^T and FME61 strains), are proposed.

Description of *Psychrobacter casei* sp. nov.

Psychrobacter casei sp. nov (ca.se'i. L. gen. n. *casei* of cheese, referring to origin of isolation).

Cells are Gram-negative, cocci or short rods, non-motile, aerobic, catalase and oxidase positives. Colonies are cream, non-translucent and raised with entire margin. Colonies grown on TSA medium at 25°C for 72h have a size of 2 mm in diameter. The strains growth occurs between 10 to 30°C, but not at 4 and 37°C, in a pH range of 6.0 to 10.0 and in the presence of 0.5 to 12% (w/v) NaCl, but not at 0 and 15%. Strains are weakly positive for reduction of nitrate to nitrite and for esterase (C4). The strains of this group are positive in the following biochemical testes: esterase lipase (C8), leucine arylamidase and naphthol-AS-BI-phosphohydrolase. The G+C content of DNA is 42.9 mol% and the genome size is near to 3.4 Mb.

The type strain FME6^T was isolated from soft-ripened goat's milk cheese rind from Mediterranean region in France.

Description of *Psychrobacter translucens* sp. nov.

Psychrobacter translucens (trans.lu'cens L. part. adj. *translucens*, being translucent).

Cells are Gram-negative, cocci or short rods, non-motile, aerobic, catalase and oxidase positive. Colonies are translucent and raised with entire margin. Colonies grown on TSA medium at 25°C for 72h have a size of 1 to 1.5 mm in diameter. The strains growth occurs between 10 to 30°C, but not at 4 and 37°C, in a pH range of 6.0 to 10.0 and in the presence of 0.5 to 12% (w/v) NaCl, but not at 0 and 15%. Strains are negative for reduction of nitrate to nitrite and weakly positive for naphthol-AS-BI-phosphohydrolase. The strains of this group are positive in the following biochemical testes: esterase (C4), esterase lipase (C8) and leucine arylamidase. The G+C content of DNA is 41.8 mol% and genome size is about 3.6-3.7 Mb.

The type strain FME13^T was isolated from soft ewe's milk cheese rind from Mediterranean region in France.

AUTHOR STATEMENTS

Authors and contributors

Caroline Isabel Kothe: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing-original draft, Writing-review & editing.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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ABBREVIATIONS

ANI: Average Nucleotide Identity

TSA: Tryptic Soy Agar

TSB: Tryptic Soy Broth

MA: Marine Agar

MB: Marine Broth

SUPPLEMENTARY MATERIAL

The supplementary Tables S1-S2 can be found at <http://dx.doi.org/10.17632/7vc3vndc3r.1>.

Table S1. Identities of 16S rRNA and *rpoB* gene sequences (%) between the cheese-associated strains investigated in the present study and closely related species. Identity values are highlighted in green when species delineation are above of the given threshold, >99% for 16S rRNA and >97.7% for *rpoB* gene sequences. **(A)** *P. casei* **(B)** *P. translucens*.

(A) *P. casei*

Species and/or Strains	16S rRNA gene		<i>rpoB</i> gene	
	FME6 ^T	FME5	FME6 ^T	FME5
<i>P. casei</i> FME6 ^T		99.94		100
FME5	99.94		100	
<i>P. aquimaris</i> SW-210 ^T	99.11	99.04	90.61	90.61
<i>P. fozii</i> NF23 ^T	99.11	99.04	89.78	89.78
<i>P. faecalis</i> Iso-46 ^T	97.61	97.54	89.03	89.03
<i>P. glaciei</i> B1c20019 ^T	99.18	99.11		

(B) *P. translucens*

Species and/or Strains	16S rRNA gene		<i>rpoB</i> gene	
	FME13 ^T	FME61	FME13 ^T	FME61
<i>P. translucens</i> FME13 ^T		99.42		100
FME61	99.42		100	
<i>P. aquimaris</i> SW-210 ^T	98.63	99.25	90.2	90.2
<i>P. fozii</i> NF23 ^T	97.95	98.56	91.36	91.36
<i>P. nivimaris</i> 88/2-7 ^T	98.63	98.84		

Table S2. Metadata related to *Psychrobacter* strains whose whole-genome sequences were used to build the phylogenomic tree in Fig. 3.

Species	Strain	Type strain	Isolate from	Country/ Region
<i>Psychrobacter alimentarius</i>	PAMC 27889	No	Rocky desert	Antarctica
<i>Psychrobacter aquaticus</i>	CMS 56	Yes	Cyanobacterial mats	Antarctica
<i>Psychrobacter aquimaris</i>	DSM 16329	Yes	Sea water	Republic of Korea
<i>Psychrobacter arcticus</i>	273-4	Yes	Permafrost soil	Russia
<i>Psychrobacter cibarius</i>	DSM 16327	Yes	Jeotgal, seafood	Republic of Korea
<i>Psychrobacter cryohalolentis</i>	K5	Yes	Cryopeg within permafrost	Russia
<i>Psychrobacter faecalis</i>	DSM 14664	Yes	Bioaerosol	Germany
<i>Psychrobacter fozii</i>	CECT 5889	Yes	Sediment	Antarctica
<i>Psychrobacter frigidicola</i>	ACAM 304	Yes	Ornithogenic soil	Antarctica
<i>Psychrobacter immobilis</i>	DSM 7229	Yes	Poultry carcass	Unknown
<i>Psychrobacter lutiphocae</i>	DSM 21542	Yes	Feces of a seal	Germany
<i>Psychrobacter namhaensis</i>	DSM 16330	Yes	Sea water	Republic of Korea
<i>Psychrobacter nivimaris</i>	LAMA 639	No	Deep-sea sediment	South Atlantic Ocean
<i>Psychrobacter pacificensis</i>	DSM 23406	Yes	Seawater	Japan
<i>Psychrobacter pasteurii</i>	CIP 110853	Yes	Human origin	France
<i>Psychrobacter phenylpyruvicus</i>	DSM 7000	Yes	Human blood	Belgium
<i>Psychrobacter piechaudii</i>	CIP 110854	Yes	Human origin	France
<i>Psychrobacter piscatorii</i>	LQ58	No	Deep-sea hydrothermal vent sediment	East Pacific Rise
<i>Psychrobacter proteolyticus</i>	HAMBL_2948	No	Unknown	Unknown
<i>Psychrobacter pygoscelis</i>	I-STPP5b	Yes	Penguin	Chilean Antarctic
<i>Psychrobacter sanguinis</i>	SM868	No	Sea lion	New Zeland
<i>Psychrobacter urativorans</i>	R10.10B	No	Soil sample	Antarctica
Nouvel cheese strains from this study				
<i>Psychrobacter casei</i>	FME5	No	Semi-soft blue cheese rind	France
<i>Psychrobacter casei</i>	FME6	Yes	Soft-ripened goat's milk cheese	France
<i>Psychrobacter translucens</i>	FME61	No	Soft smear-ripened cheese rind	France
<i>Psychrobacter translucens</i>	FME13	Yes	Soft ewe's milk cheese rind	France
Outgroup				
<i>Acinetobacter calcoaceticus</i>	DSM 30006	Yes	Soil	Netherlands
<i>Moraxella lacunata</i>	CCUG 4441	Yes	Human conjunctivitis	France

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3.4. Conclusion

Toutes les souches d'*Halomonas* et de *Psychrobacter* étudiées ici diffèrent des espèces décrites précédemment par un ANI<95-96% et possèdent quelques caractéristiques biochimiques, enzymatiques et physiologiques spécifiques. Parmi le genre *Halomonas*, ont été identifiés quatre nouvelles espèces. En plus des caractéristiques génomiques, nous avons attribué un groupe de souches à *Halomonas citridevorans*, les identifiant comme souches fromagères, capables d'assimiler le citrate de sodium, et un autre groupe à *Halomonas minus*, car elles présentent des diamètres relatifs plus petits que les autres espèces. De plus, quatre souches fromagères partagent leurs valeurs ANI, dans la zone limite d'identification d'une nouvelle espèce (95-96%) avec *H. alkaliphila* DSM 16354^T. Cependant, comme ces souches présentent une combinaison de réactions qui les différencient de la souche type environnementale, nous les avons rapportées à *Halomonas casei*. Enfin, deux autres souches fromagères, avec colonies jaunes, ont été attribuées à *Halomonas flavum* bien que distantes génétiquement des espèces existantes. Concernant le genre *Psychrobacter*, nous avons identifié deux nouvelles espèces. Un groupe de souches, distantes génétiquement de toutes les espèces identifiées, a été défini comme *Psychrobacter casei*, et se distingue de l'espèce la plus proche par l'absence de certaines enzymes. De même, deux autres souches fromagères de *Psychrobacter*, appartenant à un groupe taxonomiquement distant de ceux de la base de données, ont été assignés sous l'appellation *Psychrobacter translucens*, puisqu'elles possèdent des colonies translucides.

La définition de ces nouvelles espèces aide à catégoriser les microorganismes afin de pouvoir transmettre plus facilement les informations biologiques, et rendre compte de la diversité des espèces présentes dans les fromages. Les cinq autres nouvelles espèces potentielles, isolées lors de nos études précédentes, n'ont pas encore été caractérisées et méritent d'être valorisées lors de futurs travaux, car elles sont largement répandues dans les croûtes de fromage ([Publication 1](#)).

4. Propriétés fonctionnelles des souches halophiles alimentaires et environnementales dans un modèle fromage-agar

4.1. Contexte

Au-delà de l'isolement d'espèces fromagères, les travaux réalisés ici (incluant l'article en annexe, réalisé en partenariat avec l'Ifremer, [Publication 6](#)), ont montré que certaines souches alimentaires sont très proches, phylogénétiquement, des souches environnementales, principalement d'origine marine. Par ailleurs, étant donné la faible quantité d'informations dans la littérature sur les fonctions des protéobactéries halophiles dans le fromage (mentionné dans l'introduction bibliographique, [section 3.2](#)), et les controverses de certains auteurs qui les identifient comme des altérations alimentaires ou indicateurs de manque d'hygiène, nous avons décidé de progresser sur la connaissance des impacts technologiques que ces bactéries peuvent générer dans les fromages. Ainsi, nous avons rassemblé (donations, achats dans des collections de cultures ou isolées au cours de ce travail de thèse) une collection de 31 souches alimentaires et 12 souches environnementales des genres *Halomonas*, *Pseudoalteromonas* et *Psychrobacter* afin d'évaluer leurs fonctions éventuelles dans un modèle de fromage synthétique. Par conséquent, l'objectif de ce travail consiste à :

- évaluer la croissance de 43 souches halophiles, individuellement, dans un modèle caillé-agar ;
- estimer les propriétés fonctionnelles produits par les souches alimentaires et environnementales ;
- identifier les voies métaboliques potentielles, utilisés par ces souches dans le modèle construit.

Les résultats obtenus sont présentés sous la forme d'un article en cours de préparation et constituant la partie 4.2.

4.2. Publication 5

Functional properties of food and environmental halophilic gamma-proteobacteria strains on cheese-agar models

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Abstract

In this study, we investigated the technological impact of 43 food and environmental halophilic strains belonging to the genera *Halomonas*, *Pseudoalteromonas* and *Psychrobacter* on cheese-agar plates. Each strain was inoculated ($\sim 6 \log \text{CFU}/\text{cm}^2$) on the synthetic cheese model, individually, and the plates were incubated at 15°C during 21 days. After this time, we performed sensory analyses (sniffing, visual color and textures) as well microbiological counts, pH measures and quantification of volatile compounds. Results showed that several strains have an intensity of aroma perceived by evaluators and some of them changed the visual aspect on the surface of the plates. Additionally, the majority of strains tested were able to grow on the cheese-agar, including the environmental strains, and several of them are able to change the pH of the medium (increase or decrease). Particularly, four cheese strains were emphasized concerning the production of volatile compounds. Among them, two *Psychrobacter* strains (*Psy. casei* FME6 and *Psy. namhaensis* 1439) generated a variety of carboxylic acids and two strains of *Pseudoalteromonas nigrifaciens* (FME68 and FME53) produced a variety of pyrazines, methyl ketones and sulphur compounds, which may contribute to the aroma of cheeses. This study is a first approach of investigation of the functional properties effect of a large collection of halophilic strains on cheese. It may serve as a basis for choosing strains that could have an impact on the sensory quality of cheeses, in order to carry out more in-depth analyses and explore the interactions with other bacteria and yeasts present in this fermented food.

Keywords: aroma compounds, gram-negative, cheese rinds, halophiles

1 Introduction

Currently, over 1,400 different types of cheeses are produced in various parts of the world, with a wide variety of textures, appearance, flavours and aromas. In addition to local and technological factors, these characteristics are due to the development of complex and specific microbial communities, which can be added as starter or ripening cultures or can be inserted incidentally along the production chain [1,2]. Lactic acid-bacteria (LAB) are the main microorganisms found inside cheeses and have been well studied for their functional properties, either by acidifying the medium or by producing aroma compounds [3,4]. However, the microbiota of the cheese surface is more diverse and complex, and can be influenced by the ripening room, equipments, materials and ingredients used in production. Indeed, Wolfe *et al.* (2014) showed that around 60% of bacterial genera present in cheese rinds are adventitious [5]. In recent years, metagenomic and amplicon-sequencing studies showed that certain marine halophilic genera, such as *Halomonas*, *Pseudoalteromonas* and *Psychrobacter*, can be abundant in cheese communities, especially in washed-rinds [5-7], suggesting they may have an impact on the organoleptic properties of cheeses.

Although Gram-negative bacteria have been often associated with food as contaminants or causing spoilage, some studies showed that they could play an important role in cheese ripening, contributing to its organoleptic characteristics. Some authors suggested, for example, that the presence of *Halomonas* spp. was considered an indicator of hygienic deficiencies in cheese production facilities [8], while others suggested that some species can play an important role in cheese ripening, especially for cheeses washed with brine [7,9]. Concerning *Pseudoalteromonas* species, it was recently established that some genomes of strains associated to the cheese habitat encode lipases and proteases adapted to the cold, which may contribute to the production of flavour compounds, as well as the MGL gene (methionine gamma-lyase), which transforms methionine into methanethiol, a sulphur compound [5]. Finally, it has been reported that some *Psychrobacter* strains produce significant amounts of volatile compounds [10-12], which may contribute to the flavour of cheeses. However, in general, the ability of halophilic Gram-negative strains to be used as cheese ripening component and their contribution to colour, texture and flavour is still poorly understood.

Recently, Kothe *et al.* (2020) performed a systematic isolation of halophilic strains in cheese rinds, obtaining a collection of diverse species [13]. The isolation of such a representative set of strains opens the opportunity to explore the physiological characteristics, as well as the potential functional properties of these bacteria in cheese technology. Here, we gathered a collection of cheese strains of the genera *Halomonas*, *Psychrobacter* and *Pseudoalteromonas*, as well as closely related strains from other environments, to evaluate their characteristics, such as growth capacity, pH changes and the production of volatile compounds, on the surface of cheese-agar models and discuss their possible roles in cheese technology.

2 Methodology

2.1 Halophilic strains and culture preparation

In this study, we analysed 43 food and environmental halophilic strains belonging to the genera *Halomonas*, *Psychrobacter* and *Pseudoalteromonas* (Table S1). Most of the cheese isolates were obtained from previous studies exploring the diversity of halophilic bacteria in artisanal cheeses [13,7]. The other strains were acquired via donations or bought from DSMZ (German Collection of Microorganisms), and are phylogenetically closely related to the cheese strains.

From a stock at -80°C, each strain was grown in 14 ml tubes containing 2 ml of Tryptic Soy Broth (TSB, Sigma Aldrich)+2% NaCl medium for 72 h at 28°C under aerobic condition (120 rpm). Afterwards, 0.2 ml of this medium was inoculated in a second preculture containing 10 mL of TSB+2% salt in a 50 ml erlenmeyer flask closed with cellulose stoppers and incubated 72 h at 28°C under aerobic condition (200 rpm). Then, the cells were recovered by centrifugation and washed with physiological water (NaCl 9g/L).

2.2 Cheese-agar model and strain inoculations

The unsalted and unripened curd (pH~5), used for the cheese model production, was provided by an industrial cheesemaker. It was produced from pasteurized milk using a mesophilic starter culture composed of *Lactococcus* strains, and according to the typical Raclette-type manufacturing process. Blocks of approximately 500g were packed in plastic bags and stored at -20°C, until use. The defrosted cheese (24 hours at 4°C) was grated with an electric grater and a saline solution (NaCl 35.4 g/L) was added to the curd to obtain a final concentration equivalent to 1.7% of NaCl. The mixture was then homogenized with a mechanical blender (Ultra-Turrax model

T25, IKA Labortechnik, Staufen, Germany) at 24,000 tr/min for 8 min. A volume of 5 M NaOH equivalent to 14 ml per kg of salted curd mixture was added progressively to increase the pH of the medium, and the content was mixed again for a 3 min period. Finally, agar (Type E agar, Biokar Diagnostics, Beauvais, France) was added to a final concentration of 10g/L and the mixture was transferred in 500-mL bottles which were autoclaved at 110°C for 10 min. The subsequent stages of cheese model production were carried out under aseptic conditions. A volume of about 40 ml of the molten cheese-agar (65°C) was poured into petri dishes (8.5 cm in diameter) and allowed to solidify at room temperature.

A volume of 100 µl of halophilic strain suspension was inoculated onto the cheese-agar plates using glass beads, representing an inoculation rate of about of 10⁶ CFU/cm². For each strain, nine inoculated cheese-agar plates were incubated for 21 days at 15°C. Three of these plates were used for microbiological analyses, three for quantifying flavour compounds and the last three for sensory qualitative analysis. Blank uninoculated cheese-agar plates were incubated under the same conditions.

2.3 Determination of bacterial growth

The surface biomass of the cheese-agar plates after 21 days of incubation was recovered (2-3g) with sterile spatulas and transferred in physiological water (9g/L NaCl). After homogenization with an Ultra-Turrax blender for 1 min at 24,000 tr/min, 10-fold serial dilutions were prepared in physiological water, and spread in duplicate on TSA+2% NaCl agar. Colonies were enumerated after 72 h of incubation at 28°C.

2.4 Qualitative sensory description

Twelve non-trained panellists from our laboratory staff carried out qualitative aroma description of cultures inoculated on cheese-agar models. The participants sniffed the non-inoculated plates and the samples after 21 days of incubation, and evaluated the intensity of the smell between 0 (corresponding to the non-inoculated plates) and 10 for each strain, and choose their own descriptors to define the odours perceived. They also described other characteristics of each strain, such as colour and aspect changes on the surface of the cheese-agar model.

2.5 Sampling for volatile compound analysis and pH measurements

The sampling to quantify the volatile compounds produced by the halophilic strains in the cheese-agar models were carried out in a cold room (4°C) to prevent the loss of volatile compounds. The cheese-agar of each plate was well homogenized and approximately 7g of sample were then transferred into sterile amber glass bottles, which were subsequently frozen at -80°C. The remaining of the samples was used to measure the pH values, with a standard pH-meter (Jenco Vision 6071, San Diego, CA, USA).

2.6 Identification of volatile compounds by GC-MS

To identify the aroma compounds present in the non-inoculated and inoculated cheese agar models, GC/MS analysis was performed. These analyses were only realized for the strains with high aromatic intensity perceived by the evaluators in sniffing analysis. Volatile compounds were extracted using the purge and trap method by means of a Gerstel Dynamic Headspace System (DHS) coupled with a Gerstel Multipurpose Sampler (MPS) Autosampler (Mulheim an der Ruhr, Denmark) as described previously [14]. Nevertheless, in this study, only three grams of the samples were weighed in a vial.

2.7 Statistical analyses

Barplot and boxplot were elaborated with ggplot package of R (v. 3.6.3) to depict the microbial counts and pH values, respectively. Statistical analysis for pH values were performed via examination of variance (ANOVA) applying the Tukey test ($p < 0.05$). The function multcompLetters from multcompView package of R was used to assign different letters to the group exhibiting statistically significant difference. The heatmap with participants' evaluations at sniffing analysis were elaborated using pheatmap package of R. Furthermore, the Principal Coordinates Analysis (PcoA) of volatile compounds detected in the samples was build using the 'FactoMineR' package [15].

3 Results

3.1 Choice of representative panel of strains

For this study, we collected a variety of species and strains belonging to the genera *Halomonas*, *Pseudoalteromonas* and *Psychrobacter*, from different habitats (Fig. 1 and Table S1). However, we focused our collection on a set of 43 strains of the variety of species found in cheese (29 strains), as determined in previous studies [7,13], and completed with several genetically related strains isolated from other food products (3 strains) or from environmental sources (12 strains).

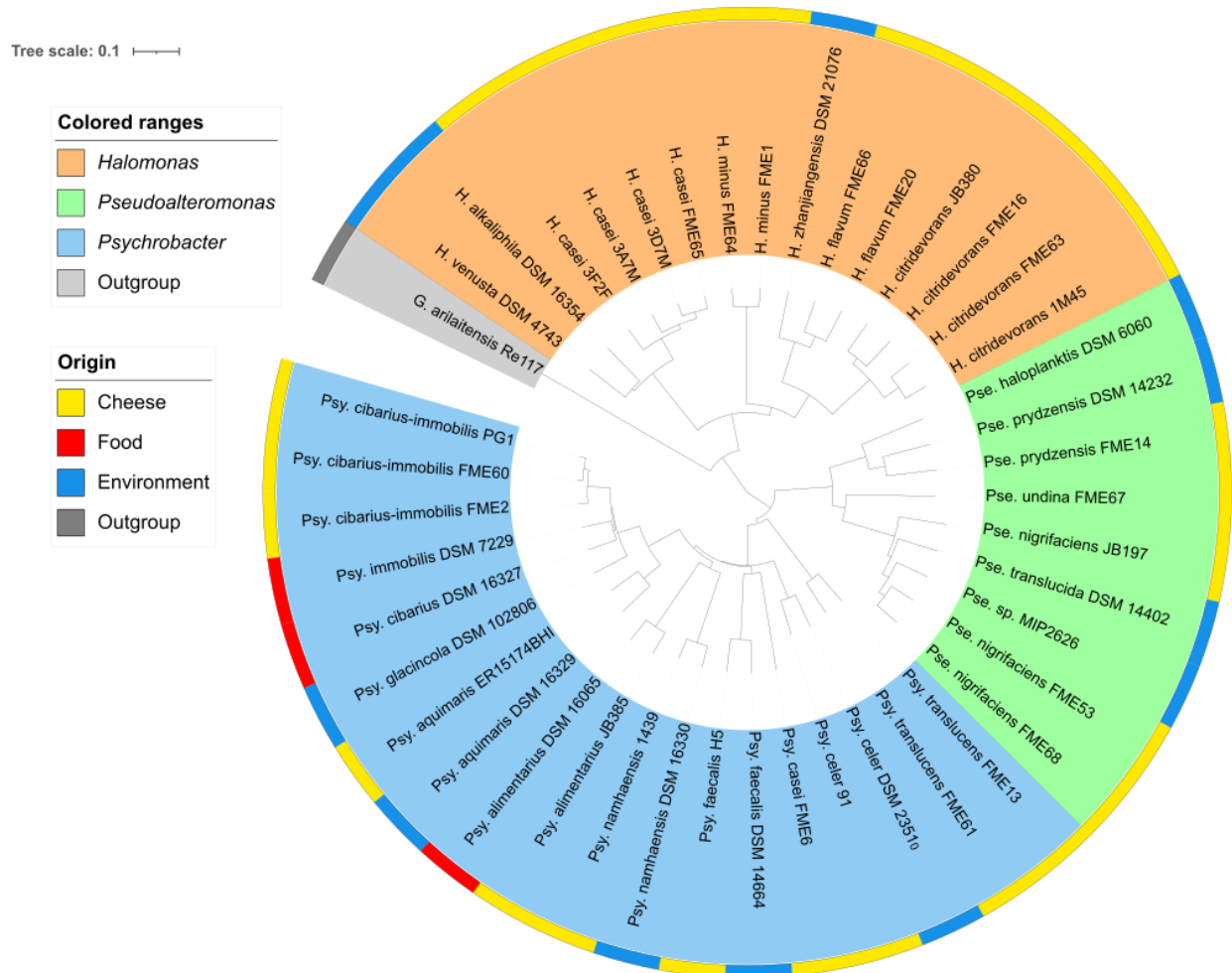


Fig. 1. Phylogenomic tree of 43 halophilic strains used in this study.

As shown in [Figure 1](#), based on ANI analysis ([Table S2](#), considering 95.5% of ANI cut-off), these strains may be grouped in 19 potential species. The genus *Halomonas* is represented by four species isolated from cheese, with two to four strains, and three strains related species originating from marine environment. Similarly, *Pseudoalteromonas* are represented by three species with one to three strains isolated from cheese, and four strains from marine environment. Lastly, *Psychrobacter* is the largest group and contains strains from eight species isolated from cheese, three strains from other type of food and five strains from marine environment. Nevertheless, this panel of strains was further assessed to test their ability to develop in cheese-agar model and produce metabolic compounds of interest for cheese technology.

3.2 Growth and pH changes in the cheese-agar model

The bacterial counts after 21 days of incubation are shown in [Fig. 2A](#). Overall, we observed that most halophilic strains were able to grow on the cheese-agar model, including the environmental strains. The average cell counts was between 6.5 and 9.6 log CFU/cm², except for three *Pseudoalteromonas* strains, one of cheese origin (JB197) and two isolated from the sea (DSM 14232 and DSM 14402), which counts were <6 log CFU/cm². Concerning *Halomonas* strains, most of them alkalise the cheese-agar, including three environmental strains ([Fig. 2B](#)). Several *Psychrobacter* strains (six) slightly acidify the model (especially 1439, FME6 and DSM 16329), while three strains - belonging to different species - considerably increase the pH. Moreover, two of the *Pseudoalteromonas* strains increase the pH, corresponding to the strains of this genus that has the greatest development on cheese-agar ([Fig. 2A](#)).

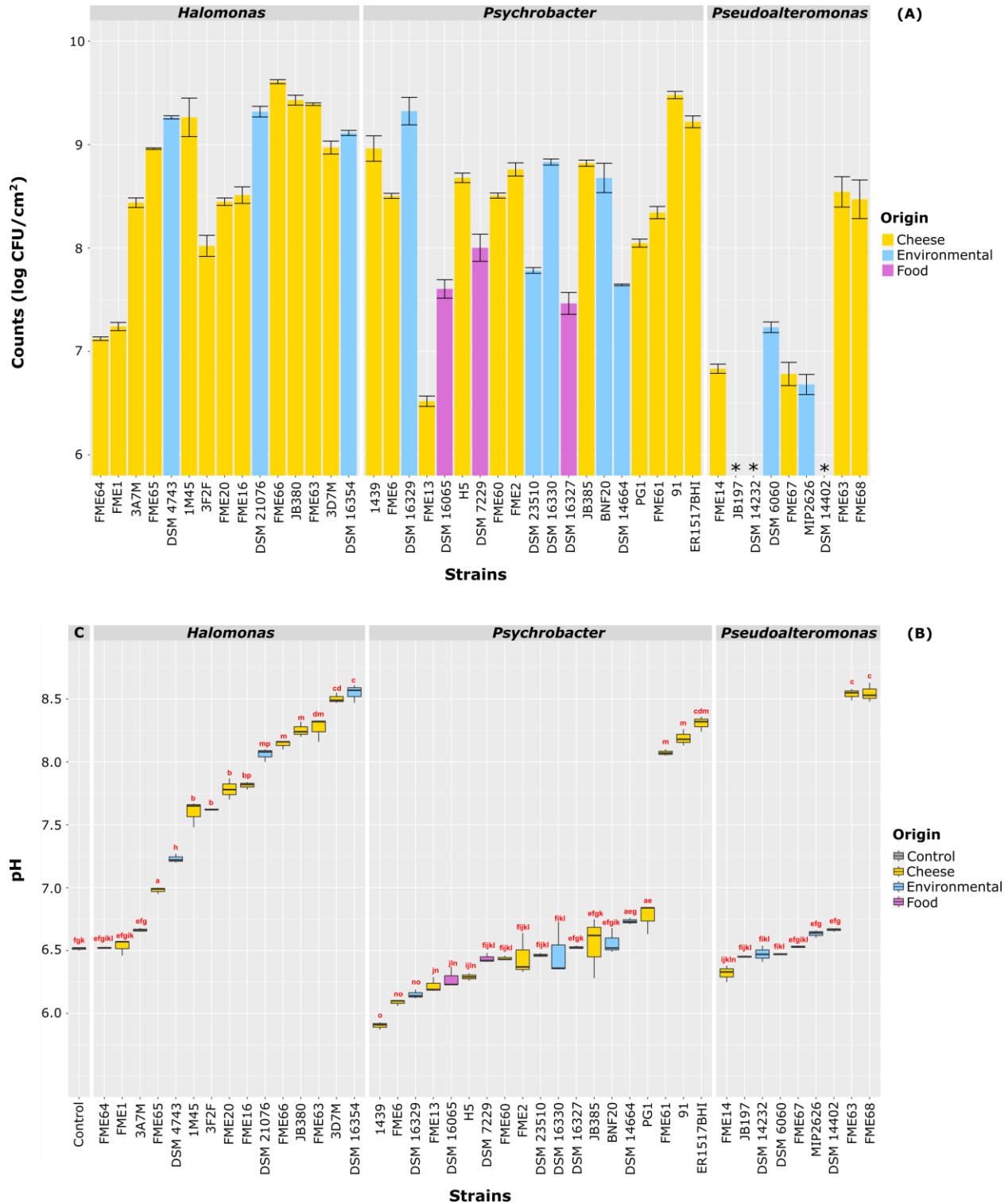


Fig. 2. Halophilic bacterial counts (A) and pH measures (B) at day 21 on cheese-agar model. The error bars represent the standard deviations (three replicates) and the asterisks indicate the low cell counts lower than 6 log CFU/cm² (A). The different letter indicate a significant difference in pH (p<0.05) (B).

3.3 Sensory analysis

We conducted a sensory analysis where non-trained panellists scored the intensity of the odours perceived for each strain, and described them qualitatively, as well as the visual aspects observed. It resulted in the formation of two clusters that separated the intense aromatic strains from poor, according to the scores of evaluators' sniffing (Fig. 3). Based on these results, we observed that, overall, the *Halomonas* strains showed less perceived odours, while the *Psychrobacter* strains, whether from food or environmental origins, tended to display more intense. Additionally, two cheese strains of *Pseudoalteromonas* deserve emphasis (FME68 and FME53), as they have been described by all evaluators as having a strong odour (descriptors such as ammonia, ripened cheese rind and sour are cited). Moreover, they exhibited a brown colour with white spots on the surface of the cheese-agar models (Additional File 1). We also observed that several strains formed a biofilm on the surface of cheese-agar medium with white spots (*Pse. prydzensis* FME14 and *Psy. translucens* FME13) or with slightly yellow colour (FME66 and DSM 21076 *Halomonas* strains). Finally, several *Psychrobacter* strains formed a white biofilm and wrinkled textures (especially highlighted in DSM 16065, JB385, PG1, FME2, FME60 and DSM 102806). These observations suggest that several halophilic strains may have potential contributions for visual aspects on cheese rinds.

3.4 Production of volatile compounds

From the above-mentioned results, especially from sniffing (Fig. 3), 37 strains were selected to evaluate their potential production of volatile compounds. Overall, we decided to perform a triplicate analysis for the cheese strains with higher notes in sniffing analysis and only one replicate for the others and for some environmental strains, particularly those with the highest aroma intensity. We also evaluated the volatile compounds generated for the controls (without bacterial inoculum) (i) after 21 days of incubation and (ii) before incubation; totalling 71 samples (replicates are described in Table S3). We identified a total of 582 molecules produced by GC-MS analysis. In order to reduce these volatile compounds to odour-active compounds, a filter was applied using the reference site: www.thegoodscentscompany.com and the literature. Finally 216 aromatic molecules were kept.

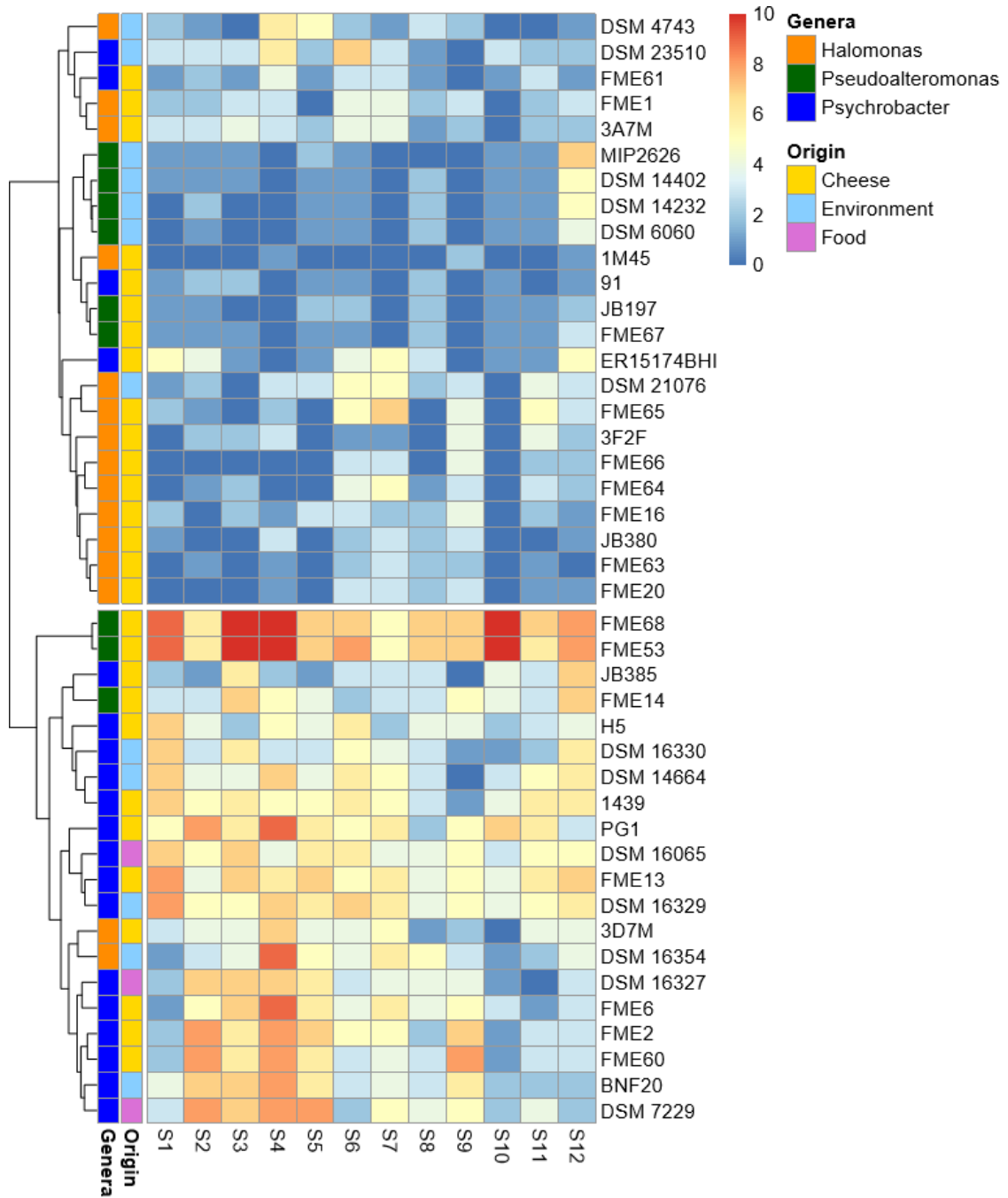


Fig. 3. Heatmap depicting the intensity perceived in sniffing analysis by twelve non-trained panelists.

Figure 4A highlights two groups with particular aromatic characteristics. The first one was composed by *Pse. nigrifaciens* FME68 and FME53 strains, which produced volatile compounds such as pyrazine, ketones (acetone, 2-butanone, methyl isobutyl ketone, etc), benzaldehyde and dimethyl disulfide, that can contribute to the aroma in cheese ripening [16]. The second group with

attractive characteristic aromas included mainly the cheese strains of *Psy. casei* (FME6) and *Psy. namhaensis* (1439) (Fig. 4B), which produced carboxylic acid compounds such as 2-methyl butanoic acid, 3-methyl butanoic acid, decanoic acid, 2-methyl propanoic acid and acetic acid. In addition of these carboxylic acids, they also produced ketones such as 2,3-butanedione, acetoin, 2-3 pentanedione, 2H-pyran-2-one tetrahydro-6-propyl and 2H-pyran-2-one tetrahydro-6-methyl. In order to better explore other halophilic strains with a potential flavouring role in cheeses, statistical analyses were carried out for each genus, separately (Fig. S1). Although Fig. 4B clustered all *Halomonas* strains with the controls (without bacteria inoculation), we observed here that they presented distinct groups (Fig. S1-A). For example, DSM 16354 strain displayed a different volatile compound profile, with emphasis on the production of pyrazines, ketones and sulphur compounds. Indeed, this strain had the most intense odour (among *Halomonas*) in the sniffing analysis (Fig. 3), and odors of mushroom, soil, plant and vegetables were described, consistent with the description of ketone and pyrazine compounds detected (Table S4). Similarly, *Pseudoalteromonas prydzensis* FME14 presented different volatile compound profile from the control, with production of acetoin and 2-propanone, 1-hydroxy compounds (Fig. S1-B). Additionally, this strain also generated white spots on the biofilm and was described by the evaluators as containing the odour of milk and cheese. Finally, we observed also other group of *Psychrobacter* strains that deserve emphasis, which include the strain JB385 and two strains of environmental origin (DSM 16065 and DSM 16330) (Fig. S1-C). These strains produced especially ester and alcohol molecules and the evaluators noted aromas of Parmesan, Gruyère and Emmental cheeses in the sniffing analyses. The result of the quantification of the 216 volatile compounds in the 71 samples, as well the odour description of each molecule, can be visualised in Table S4.

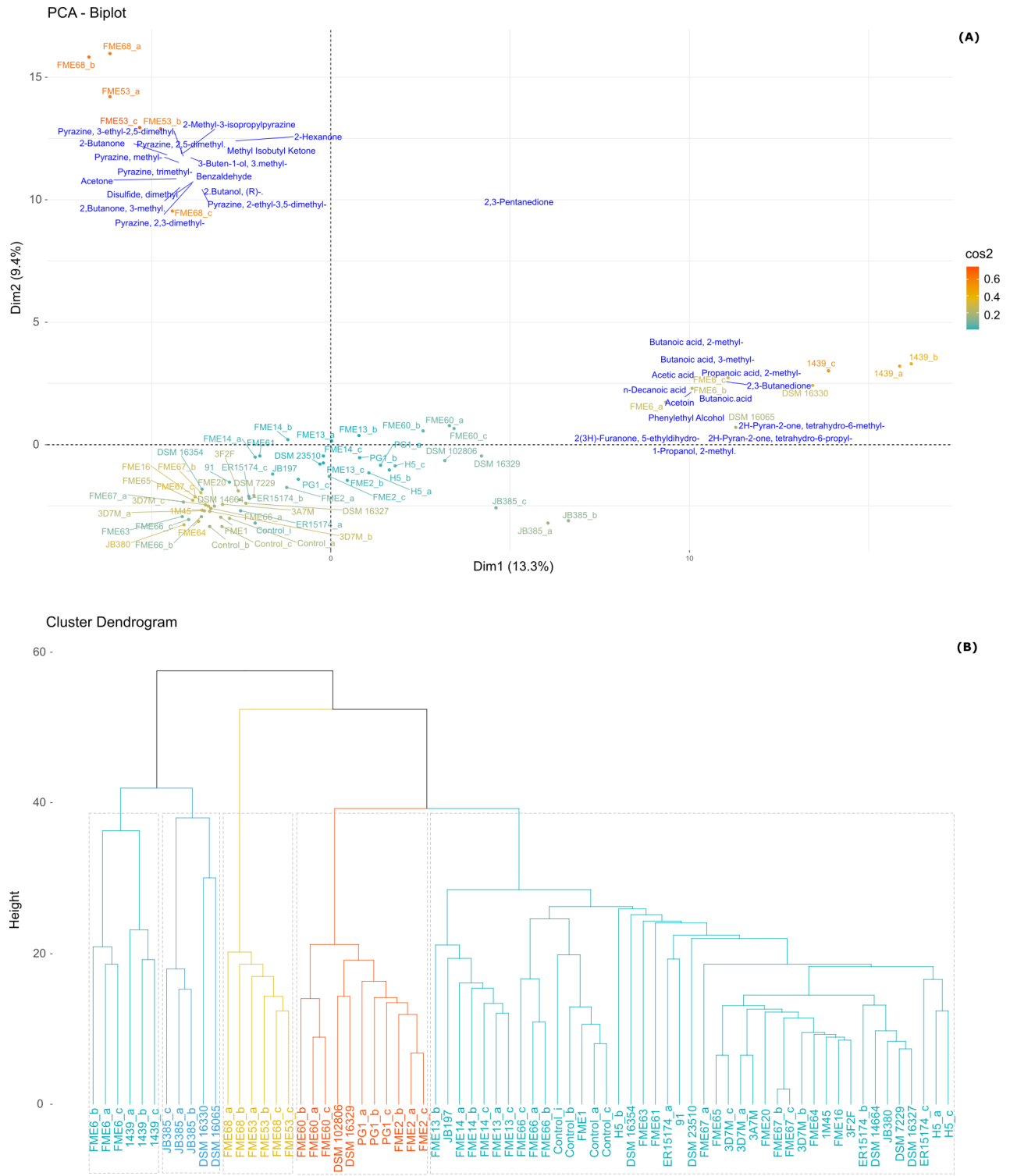


Fig. 4. Overview of volatiles profiles of 43 halophilic strains on cheese-agar models. **(A)** Principal component analysis (PCA) showing the mainly aromatic compounds produced by different group of strains (cos2: square cosine). **(B)** Clustering samples by similarity of aromatic profiles.

4 Discussion

A few number of studies, until now, has focused in the investigation of Gram-negative bacteria during cheese ripening [11,17,10]. Here, we collected a large number of strains of *Halomonas*, *Pseudoalteromonas* and *Psychrobacter*, to test their functional properties on a cheese-agar model.

Overall, most of the cheese and environmental strains were able to grow on the proposed model (Fig. 2A). Additionally, the three genera evaluated have strains that are capable of increasing the cheese-agar pH (Fig. 2B), and this alkalisation may be related to the consumption of lactic acid from the curds and/or to their strong proteolytic activity [18]. Interestingly, six strains of *Psychrobacter* and one of *Pseudoalteromonas* presented pH slightly lower than the control, and white spots were observed on the cheese-agar surfaces of two of them: *Psy. translucens* FME13 and *Pse. prydzensis* FME14 (Additional File 1). These spots may be associated with the formation of calcium lactate crystals, which may be formed in cheeses with low pH due the development of insoluble lactate [19]. Finally, we have not observed any clear correlation between the growth of the strains and the evolution of the pH on the model surface, since several strains presented counts above 8 log CFU/cm² and no pH changes.

Among the 43 strains evaluated, two groups were distinguished by their production of volatile compounds. The first one, composed of *Pse. nigrifaciens* FME68 and FME53 strains, showed significant production of pyrazines in the cheese-agar model (Fig. 4A), that can be explained by isomerization of the aminoacetone derived from the degradation of alanine and methylglyoxal (Strecker degradation) [20]. The formation of different pyrazines was described in Parmesan cheeses and associated with a brown discoloration [20]. Interestingly, we observed a brown color on the cheese-agar surfaces from these strains, which could be associated with the pyrazine compounds generated by them (Additional File 1). Pyrazines were also reported to display baked, roasted, and nutty flavor characters [21]. Moreover, both *Pseudoalteromonas* strains also produced ketones, which can be formed from the fatty acids in the cheese by beta-oxidation and can generate secondary alcohols. These compounds have been commonly reported in blue cheeses [22,23]. Benzaldehyde, another compound generated by the two strains, was probably formed from phenylpyruvic acid, derived from the aminotransferase activity in phenylalanine [24] and has already been described as having an aromatic note of bitter almond and reported in the volatile fractions of several cheeses, such as Brie and Camembert [25,26].

Additionally, FME68 and FME53 strains had high levels of disulfide dimethyl, a volatile compound formed from methanethiol, one of the degradation products of L-methionine metabolism. This sulphur compound was already detected in cheeses such as Camembert, Livarot, Langres and Epoisses [27-29], and can bring aromatic notes of cabbage, garlic and cheesy. Although evaluators in sniffing analysis did not perceive nutty and almond flavours, all of them highlighted an intense sour and pungent smell to the nose for both strains, and described ammonia and ripened cheese odours, which may be related to proteolytic activity of FME68 and FME53 strains. This production of ammonia may mask the perception of other odours.

The other group, composed by FME6 and 1439 *Psychrobacter* strains, showed intense production of carboxylic acids and ketones compounds (Fig. S1-C). Among the carboxylic acids, the butanoic acid, 2-methyl and butanoic acid, 3-methyl (isovaleric acid), originate from isoleucine and leucine catabolism, respectively, are branched-chain volatile acids associated to fruity aromatic notes of Comté, Abondance and Swiss cheeses [30-33]. On the other hand, FME6 and 1439 strains produced decanoic acid as one of the most prevalent fatty acid, a compound which was reported with high abundance in semi-hard and hard goat cheeses [34,35], but related as undesirable volatile compound found in Parmesan cheese due to their soapy and waxy aromas [36]. Although fruity cheeses such as Parmesan Grueyère and Emmental cheeses were mentioned by several evaluators in the sniffing analysis, other ones mentioned notes of rancidity, which may be related to the identified carboxylic and fatty acids, respectively. Interestingly, these *Psychrobacter* strains slightly lowered the pH on cheese-agar models compared to the control (Fig. 2B), and can be related with the production of acid compounds such as acetic acid and propanoic acid, 2-methyl. These volatile compounds were already reported in cheeses and were associated to the vinegar and vomit-foot odors [37]. Finally, carboxylic acids can also play an essential role in the production of aromas, since they could be precursors of other volatile compounds such as ketones, alcohols, lactones, aldehydes and esters [38]. Among the ketones produced by FME6 and 1439 strains, diacetyl (2,3-butanedione) and its reduced forms (acetoin) can be produced by the metabolism of sugars via pyruvate and acetolactate synthase. These compounds are commonly produced by LAB metabolism of citrate (i.e. by *Leuconostoc mesenteroides cremoris* and *Lactococcus lactis* subsp. *diacetylactis*) and present buttery or butterscotch note in foods [39,40]. Indeed, the evaluators of the sniffing analysis noticed odours of butter for both strains.

Previous studies have also shown that *Psychrobacter* strains, such as *Psy. celer* 91 and *Psychrobacter sp.* 580, can contribute significantly to cheese ripening through the production of aldehydes, esters, ketones and sulphur compounds [10,11]. Interestingly, we also tested the strain 91, and although this strain showed good implementation on our cheese-agar model and increased the pH of the medium (Fig. 2), its production of aromatic compounds did not have large differences with the control. In the previous studies, *Psy. celer* 91 was tested on a washed cheese model in interaction with seven other bacteria and four yeasts [10]. It is thus likely that *Psy. celer* 91 requires interactions of other bacteria and yeasts to produce aroma, while in our study this strain was tested individually.

5 Conclusion

In this study, we assessed the potential of a large variety of halophilic Gram-negative strains, isolated from cheese and from different habitats to produce aroma in a model of cheese. It shows that most strains were able to develop in this cheese agar model. Moreover, we found that the potential roles of the strains differed significantly. Two strains of *Pse. nigrifaciens* displayed a high aromatic potential, which was highlighted by sensory analysis and could be associated with the production of a variety of volatile compounds. Two *Psychrobacter* strains also stand out of the rest of the strains by producing a variety of carboxylic acids and lowering the pH in the cheese-agar model. This study highlight the potential of Gammaproteobacteria, which are part of the dominant bacteria on several washed rind cheeses, to produce aroma, texture and color in cheese technology.

Acknowledgements

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Supplementary Material

The supplementary Table S1-S4 and the Additional Files 1 can be found at <http://dx.doi.org/10.17632/7vc3vndc3r.1>.

Table S1. Halophilic strains used in this study and their respective origin and references of isolation.

Sample	Genera	Species	Strain	Origin	Obtained from
1	<i>Halomonas</i>	<i>citridevorans</i>	1M45	Cheese	Almeida et al (2014)
2	<i>Halomonas</i>	<i>citridevorans</i>	FME63	Cheese	Kothe et al (2021)
3	<i>Halomonas</i>	<i>citridevorans</i>	FME16	Cheese	Kothe et al (2020)
4	<i>Halomonas</i>	<i>citridevorans</i>	JB380	Cheese	Donation from Dutton lab
5	<i>Halomonas</i>	<i>flavum</i>	FME20	Cheese	Kothe et al (2020)
6	<i>Halomonas</i>	<i>flavum</i>	FME66	Cheese	Kothe et al (2021)
7	<i>Halomonas</i>	<i>zhanjiangensis</i>	DSM 21076	Environment	DSMZ
8	<i>Halomonas</i>	<i>minus</i>	FME1	Cheese	Kothe et al (2020)
9	<i>Halomonas</i>	<i>minus</i>	FME64	Cheese	Kothe et al (2021)
10	<i>Halomonas</i>	<i>casei</i>	FME65	Cheese	Kothe et al (2021)
11	<i>Halomonas</i>	<i>casei</i>	3F2F	Cheese	Almeida et al (2014)
12	<i>Halomonas</i>	<i>casei</i>	3D7M	Cheese	Almeida et al (2014)
13	<i>Halomonas</i>	<i>casei</i>	3A7M	Cheese	Almeida et al (2014)
14	<i>Halomonas</i>	<i>alkaliphila</i>	DSM 16354	Environment	DSMZ
15	<i>Halomonas</i>	<i>venusta</i>	DSM 4743	Environment	DSMZ
16	<i>Psychrobacter</i>	<i>cibarius-immobilis</i>	PG1	Cheese	Almeida et al (2014)
17	<i>Psychrobacter</i>	<i>cibarius-immobilis</i>	FME2	Cheese	Kothe et al (2020)
18	<i>Psychrobacter</i>	<i>cibarius-immobilis</i>	FME60	Cheese	Kothe et al (2021)
19	<i>Psychrobacter</i>	<i>glacincola</i>	DSM 102806	Environment	DSMZ
20	<i>Psychrobacter</i>	<i>immobilis</i>	DSM 7229	Food	DSMZ
21	<i>Psychrobacter</i>	<i>cibarius</i>	DSM 16327	Food	DSMZ
22	<i>Psychrobacter</i>	<i>casei</i>	FME6	Cheese	Kothe et al (2020)
23	<i>Psychrobacter</i>	<i>translucens</i>	FME61	Cheese	Kothe et al (2021)
24	<i>Psychrobacter</i>	<i>translucens</i>	FME13	Cheese	Kothe et al (2020)
25	<i>Psychrobacter</i>	<i>aquimaris</i>	ER15174BHI	Cheese	Almeida et al (2014)
26	<i>Psychrobacter</i>	<i>aquimaris</i>	DSM 16329	Environment	DSMZ
27	<i>Psychrobacter</i>	<i>faecalis</i>	H5	Cheese	Almeida et al (2014)
28	<i>Psychrobacter</i>	<i>faecalis</i>	DSM 14664	Environment	DSMZ
29	<i>Psychrobacter</i>	<i>namhaensis</i>	1439	Cheese	Almeida et al (2014)
30	<i>Psychrobacter</i>	<i>namhaensis</i>	DSM 16330	Environment	DSMZ
31	<i>Psychrobacter</i>	<i>celer</i>	91	Cheese	Almeida et al (2014)
32	<i>Psychrobacter</i>	<i>celer</i>	DSM 23510	Environment	DSMZ
33	<i>Psychrobacter</i>	<i>alimentarius</i>	DSM 16065	Food	DSMZ
34	<i>Psychrobacter</i>	<i>alimentarius</i>	JB385	Cheese	Donation from Dutton lab
35	<i>Pseudoalteromonas</i>	<i>prydzensis</i>	FME14	Cheese	Kothe et al (2020)
36	<i>Pseudoalteromonas</i>	<i>prydzensis</i>	DSM 14232	Environment	DSMZ
37	<i>Pseudoalteromonas</i>	<i>haloplanktis</i>	DSM 6060	Environment	DSMZ
38	<i>Pseudoalteromonas</i>	<i>nigrifaciens</i>	JB197	Cheese	Donation from Dutton lab
39	<i>Pseudoalteromonas</i>	<i>nigrifaciens</i>	FME68	Cheese	Kothe et al (2021)
40	<i>Pseudoalteromonas</i>	<i>nigrifaciens</i>	FME53	Cheese	Kothe et al (2020)
41	<i>Pseudoalteromonas</i>	<i>sp.</i> (not defined)	MIP2626	Environment	Donation from Ifremer
42	<i>Pseudoalteromonas</i>	<i>translucida</i>	DSM 14402	Environment	DSMZ
43	<i>Pseudoalteromonas</i>	<i>undina</i>	FME67	Cheese	Kothe et al (2021)

Table S2. ANI analysis of the 43 strains used in this study.

	01_Ha	02_Ha	03_Ha	04_Ha	05_Ha	06_Ha	07_Ha	08_Ha	09_Ha	10_Ha	11_Ha	12_Ha	13_Ha	14_Ha	15_Ha
01_Halomonas_citridevorans_1M45.fasta															
02_Halomonas_citridevorans_FME63.fasta	98.61														
03_Halomonas_citridevorans_FME16.fasta	96.31	96.17													
04_Halomonas_citridevorans_JB380.fasta	95.54	95.44	97.64												
05_Halomonas_flavum_FME20.fasta	84.2	84.46	83.97	83.81											
06_Halomonas_flavum_FME66.fasta	84.54	84.62	84.04	83.76	98.33										
07_Halomonas_zhanjiangensis_DSM_21076.fasta	84.17	84.2	83.88	83.84	94.14	94.08									
08_Halomonas_minus_FME1.fasta	79.76	80.68	79.77	79.28	79.29	79.97	79.44								
09_Halomonas_minus_FME64.fasta	79.82	79.88	79.79	79.24	79.67	79.99	79.37	99.07							
10_Halomonas_casei_FME65.fasta	79.85	79.98	79.61	79.46	80.12	81.06	79.37	79.56	79.6						
11_Halomonas_casei_3F2F.fasta	79.84	80.26	79.64	79.42	80.02	80.2	79.54	79.6	79.55	99.03					
12_Halomonas_casei_3D7M.fasta	79.9	80.12	79.51	79.41	80.11	80.51	79.32	79.33	79.36	99.31	99				
13_Halomonas_casei_3A7M.fasta	80.01	79.87	79.59	79.47	80.08	80.28	79.34	79.33	79.41	98.69	98.63	98.6			
14_Halomonas_alkaliphila_DSM_16354.fasta	79.14	79.13	79.36	79.05	79.21	79.24	79.2	79.12	79.17	95.55	95.52	95.59	95.56		
15_Halomonas_venusta_DSM_4743.fasta	79.14	79.02	79.15	79.12	79.17	79.17	79.33	79.21	79.14	93.76	93.81	93.69	93.72	92.83	

	16_Ps	17_Ps	18_Ps	19_Ps	20_Ps	21_Ps	22_Ps	23_Ps	24_Ps	25_Ps	26_Ps	27_Ps	28_Ps	29_Ps	30_Ps	31_Ps	32_Ps	33_Ps	34_Ps	
16_Psychrobacter_immobilis_PG1.fasta																				
17_Psychrobacter_cibarius-immobilis_FME2.fasta	99.49																			
18_Psychrobacter_cibarius_FME60.fasta	99.64	99.57																		
19_Psychrobacter_glaciacola_DSM_102806.fasta	96.12	96.25	96.11																	
20_Psychrobacter_immobilis_DSM_7229.fasta	95.95	96.05	95.97	96.32																
21_Psychrobacter_cibarius_DSM_16327.fasta	96.13	96.21	96.15	96.35	96.28															
22_Psychrobacter_casei_FME6.fasta	82.36	82.17	82.43	82.05	82.21	82.08														
23_Psychrobacter_translucens_FME61.fasta	81.75	81.53	81.81	81.6	81.62	81.65	80.82													
24_Psychrobacter_translucens_FME13.fasta	81.84	81.79	81.94	81.74	81.81	81.6	81.52	98.61												
25_Psychrobacter_aquimaris_ER15_174_BH17.fasta	91.63	91.3	91.51	91.19	91.26	91.26	82.49	81.84	82.01											
26_Psychrobacter_aquimaris_DSM_16329.fasta	91.21	91.1	91.23	91.15	91.26	91.19	82	81.45	81.64	97.18										
27_Psychrobacter_faecalis_H5.fasta	81.86	81.79	81.89	81.9	81.96	81.91	82.22	80.23	80.8	81.94	81.45									
28_Psychrobacter_faecalis_DSM_14664.fasta	81.73	81.77	81.82	81.65	81.92	81.83	81.62	80.4	80.34	81.48	81.46	97.47								
29_Psychrobacter_namhaensis_1439.fasta	81.93	81.85	81.94	81.76	82.01	81.87	81.07	80.84	82.26	82.37	80.45	80.59								
30_Psychrobacter_namhaensis_DSM_16330.fasta	81.95	81.9	82.01	81.88	82.19	81.92	81.09	81.04	81.01	82.12	82.28	80.4	80.55	97.65						
31_Psychrobacter_celer_91.fasta	80.76	80.61	80.7	80.64	80.55	80.51	80.26	80.27	79.97	80.7	80.45	81.01	80.89	80.28	80.44					
32_Psychrobacter_celer_DSM_23510.fasta	80.64	80.69	80.69	80.52	80.84	80.53	80.24	80.6	80.33	80.53	80.72	80.92	80.86	80.89	80.92	97.54				
33_Psychrobacter_alimentarius_DSM_16065.fasta	80.85	80.9	81.03	80.95	80.89	81	80.56	80.64	80.55	81.38	81.49	80.06	80.08	81.91	81.81	80.11	80.03			
34_Psychrobacter_alimentarius_JB385.fasta	80.85	80.87	80.96	80.83	80.86	80.97	80.44	80.46	80.39	81.19	81.32	79.91	80.03	81.85	81.84	80.01	79.92	97.12		

	35_Pse	36_Pse	37_Pse	38_Pse	39_Pse	40_Pse	41_Pse	42_Pse	43_Pse
35_Pseudoalteromonas_prydzensis_FME14.fasta									
36_Pseudoalteromonas_prydzensis_DSM_14232.fasta	97.06								
37_Pseudoalteromonas_haloplanktis_DSM_6060.fasta	86.56	86.67							
38_Pseudoalteromonas_nigrifaciens_JB197.fasta	81.22	81.33	80.31						
39_Pseudoalteromonas_nigrifaciens_FME68.fasta	80.77	80.59	80.07	97.98					
40_Pseudoalteromonas_nigrifaciens_FME53.fasta	80.68	80.54	79.94	97.95	98.89				
41_Pseudoalteromonas sp. MIP2626.fasta	80.25	80.26	79.92	97.71	98.12	98.06			
42_Pseudoalteromonas_translucida_DSM_14402.fasta	80.07	80.12	80.01	96.02	96.21	96.22	96.25		
43_Pseudoalteromonas_undina_FME67.fasta	81.04	80.52	80.19	82	82.5	82.19	81.64	81.46	



Table S3. Sample replicates analysed by GC-MS.

Sample	Genera	Species	Strain	Origin	Sniffing (media)	Volatile analysis (number of replicates)
39	<i>Pseudoalteromonas</i>	<i>nigrifaciens</i>	FME68	Cheese	7.75	3
40	<i>Pseudoalteromonas</i>	<i>nigrifaciens</i>	FME53	Cheese	7.75	3
34	<i>Psychrobacter</i>	<i>alimentarius</i>	JB385	Cheese	2.916667	3
35	<i>Pseudoalteromonas</i>	<i>prydzensis</i>	FME14	Cheese	4.083333	3
27	<i>Psychrobacter</i>	<i>faecalis</i>	H5	Cheese	3.916667	3
30	<i>Psychrobacter</i>	<i>namhaensis</i>	DSM 16330	Environment	3.666667	1
28	<i>Psychrobacter</i>	<i>faecalis</i>	DSM 14664	Environment	4.5	1
29	<i>Psychrobacter</i>	<i>namhaensis</i>	1439	Cheese	4.916667	3
16	<i>Psychrobacter</i>	<i>cibarius-immobilis</i>	PG1	Cheese	5.666667	3
33	<i>Psychrobacter</i>	<i>alimentarius</i>	DSM 16065	Food	5.083333	1
24	<i>Psychrobacter</i>	<i>translucens</i>	FME13	Cheese	5.75	3
26	<i>Psychrobacter</i>	<i>aquimaris</i>	DSM 16329	Environment	5.666667	1
12	<i>Halomonas</i>	<i>casei</i>	3D7M	Cheese	3.5	3
14	<i>Halomonas</i>	<i>alkaliphila</i>	DSM 16354	Environment	3.916667	1
21	<i>Psychrobacter</i>	<i>cibarius</i>	DSM 16327	Food	4	1
22	<i>Psychrobacter</i>	<i>casei</i>	FME6	Cheese	4.5	3
17	<i>Psychrobacter</i>	<i>cibarius-immobilis</i>	FME2	Cheese	4.75	3
18	<i>Psychrobacter</i>	<i>cibarius-immobilis</i>	FME60	Cheese	4.583333	3
19	<i>Psychrobacter</i>	<i>glacincola</i>	DSM 102806	Environment	4.5	1
20	<i>Psychrobacter</i>	<i>immobilis</i>	DSM 7229	Food	4.833333	1
15	<i>Halomonas</i>	<i>venusta</i>	DSM 4743	Environment	1.916667	-
32	<i>Psychrobacter</i>	<i>celer</i>	DSM 23510	Environment	2.916667	1
23	<i>Psychrobacter</i>	<i>translucens</i>	FME61	Cheese	1.75	1
8	<i>Halomonas</i>	<i>minus</i>	FME1	Cheese	2.333333	1
13	<i>Halomonas</i>	<i>casei</i>	3A7M	Cheese	2.5	1
41	<i>Pseudoalteromonas</i>	<i>sp.</i> (not defined)	MIP2626	Environment	1.25	-
42	<i>Pseudoalteromonas</i>	<i>translucida</i>	DSM 14402	Environment	1.166667	-
36	<i>Pseudoalteromonas</i>	<i>prydzensis</i>	DSM 14232	Environment	1.083333	-
37	<i>Pseudoalteromonas</i>	<i>haloplanktis</i>	DSM 6060	Environment	0.916667	-
1	<i>Halomonas</i>	<i>citridevorans</i>	1M45	Cheese	0.333333	1
31	<i>Psychrobacter</i>	<i>celer</i>	91	Cheese	0.916667	1
38	<i>Pseudoalteromonas</i>	<i>nigrifaciens</i>	JB197	Cheese	1	1
43	<i>Pseudoalteromonas</i>	<i>undina</i>	FME67	Cheese	1	3
25	<i>Psychrobacter</i>	<i>aquimaris</i>	ER15174BHI	Cheese	2.5	3
7	<i>Halomonas</i>	<i>zhanjiangensis</i>	DSM 21076	Environment	2.583333	-
10	<i>Halomonas</i>	<i>casei</i>	FME65	Cheese	2.416667	1
11	<i>Halomonas</i>	<i>casei</i>	3F2F	Cheese	1.583333	1
6	<i>Halomonas</i>	<i>flavum</i>	FME66	Cheese	1.166667	3
9	<i>Halomonas</i>	<i>minus</i>	FME64	Cheese	1.75	1
3	<i>Halomonas</i>	<i>citridevorans</i>	FME16	Cheese	1.833333	1
4	<i>Halomonas</i>	<i>citridevorans</i>	JB380	Cheese	1.25	1
2	<i>Halomonas</i>	<i>citridevorans</i>	FME63	Cheese	1	1
5	<i>Halomonas</i>	<i>flavum</i>	FME20	Cheese	1.166667	1
					Control (t=21 days)	3
					Control (t=0)	1
					Total	71

Table S4. Quantification of volatile compounds detected in the halophilic strains and their class, odor description, formula and CAS number (**simplified**).

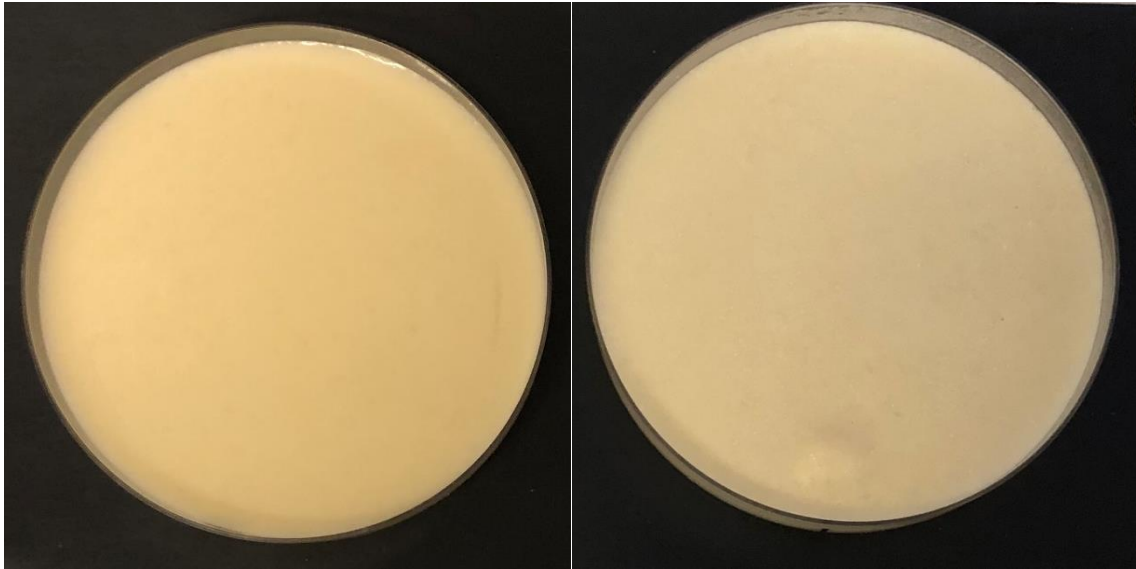
Sample	Control	FME66	3D7M	PG1	FME2	FME60	FME6	FME13	ER15174	H5	1439	JB385	FME14	FME68	FME53	FME67
1-Propanol, 2-methyl- 2,3-Butanediol	254625 0	18574.3 0	1703.33 0	506598 2326117	383797 739422	705554 566205	1393646 1089039	11586.7 0	73638.7 0	530998 0	851980 0	1052889 0	11705.3 0	34201.3 0	15685.7 0	11165.3 0
1-Butanol, 3-methyl- 1-Pentanol	803415 83684.3	33385.7 196037	0 0	1897027 0	1977240 0	3292058 0	6344694 0	167808 0	3874676 0	6458063 0	1.4E+07 0	2.1E+07 114334	210954 13436	2400584 0	3305227 0	171311 53188
2-Heptanol 2-Undecanol	502536 0	793855 0	0 0	6294708 240335	5304525 0	1.1E+07 316184	720390 0	0 0	2232768 0	693544 0	820362 0	2815117 0	0 0	1488610 0	0 0	0 0
Phenylethyl Alcohol 2-Pentanol	0 0	0 0	0 0	0 0	0 0	1414623 459401	1.2E+07 0	0 0	0 0	2486760 0	6.2E+07 0	3.9E+07 0	0 0	0 688483	0 0	0 0
1-Butanol, 2-methyl- Propanal, 2-methyl- Butanal, 3-methyl- Benzeneacetaldehyde	0 49168.7 1214233 22116.3	0 0 0 0	0 0 0 0	311504 171179 1388519 145482	171179 1078552 1129133 265117	105641 3496099 1078552 141983	2155106 4916163 376574 376574	29848.7 4916163 23726.3 23726.3	0 112471 0 0	351807 1130859 1548635 349463	434813 899510 1548635 98417.7	35502.7 899510 3188380 125208	6135.33 3188380 1961588 0	10419.7 2622457 1961588 282541	51075.7 0 2622457 263904	0 0 0 0
Acetic acid Butanoic acid	34694 0	19082.7 0	12035.3 72520.3	368738 6726919	268872 5455288	672587 2E+07	4337299 2.7E+07	4013876 4636669	50213.7 838222	992508 4645878	1.5E+07 2.4E+07	1493442 6134441	1330535 7834636	184386 3438211	841242 312005	21587 0
Butanoic acid, 3-methyl- Butanoic acid, 2-methyl- Hexanoic acid	0 0 0	0 0 0	34074.3 0 0	1.1E+07 4466313 220690	1E+07 3781490 321562	3.8E+07 8645341 1652743	3.5E+07 1.4E+07 2.4E+07	1.3E+07 2385927 1.9E+07	38652.7 0 50294.7	1.3E+07 1137284 2301640	6.3E+07 2.2E+07 1.6E+07	8017655 2922321 1767673	5234425 1353114 5424461	7679895 4320671 131921	1.1E+07 6909254 83789.3	0 0 0
Propanoic acid, 2-methyl- Octanoic acid	0 0	0 0	21838 0	3315704 0	3549414 0	1.2E+07 448682	2.6E+07 1.5E+07	3967378 4798815	0 42498.3	5088349 582681	1.8E+07 1.3E+07	3351767 1400757	0 2411003	1944489 0	1061460 115444	0 0
n-Decanoic acid Pentanoic acid Heptanoic acid Nonanoic acid Propanoic acid	0 0 0 0 0	0 0 0 0 0	0 0 0 0 0	0 0 0 0 0	0 0 0 0 0	70053.7 1088038 0 0 0	1994684 490273 212450 87772 0	546012 93783.3 0 0 137717	0 231027 0 0 0	95107.3 124088 0 0 0	3153982 1007519 251400 73390 0	183579 26687.7 0 0 0	403027 103128 0 0 0	0 0 0 0 73853.3	21576 153227 0 0 0	0 0 0 0 0
4-Methyloctanoic acid 4-Methylnonanoic acid Acetic acid, 2-phenylethyl ester	0 0 126246	0 0 17590.3	0 0 58200.3	0 0 133172	0 0 199144	0 0 395625	0 0 157136	0 86458.3 0	0 0 0	0 0 269983	0 0 1802324	0 0 1662613	0 0 0	0 0 0	0 0 76478.7	0 0 69961.7
Acetic acid, hexyl ester 2-Heptanol, acetate Isobutyl acetate Butanoic acid, methyl ester	336865 161632 90946.3 0	134524 0 0 0	108208 0 0 0	79712.3 1954652 572797 283596	67455.7 1454874 609648 371729	0 2942612 0 365639	53199 133523 0 1257559	0 0 0 0	0 401358 0 0	0 336314 499835 104653	0 283114 0 0	132956 283114 401521 221262	296527 435002 0 0	0 0 0 0	35588 0 0 187365	43917.3 0 0 0
1-Butanol, 3-methyl-, propanoate 2-Pentanol, acetate 1-Butanol, 3-methyl-, acetate	0 0 0 0	0 0 0 0	0 0 0 0	64416.3 156825 0	46075.3 0 0	102594 0 0	0 0 0	0 0 0	140599 0 2747030	224422 50256 4076108	0 0 4239149	2738871 0 9781603	0 0 0	0 0 0	0 0 0	0 0 0
Hexanoic acid, butyl ester Acetic acid, pentyl ester	0 0	0 0	0 0	0 0	0 0	0 0	51608.3 0	0 0	0 0	36570.7 171873	85651.3 0	0 125557	0 0	0 0	0 0	0 0

CHAPITRE II – RESULTATS – Publication 5

Propanoic acid, ethyl ester	0	0	0	0	0	0	0	0	0	0	0	1.5E+07	0	0	0	0
1-Butanol, 2-methyl-, propanoate	0	0	0	0	0	0	0	0	0	0	0	700781	0	0	0	0
Butanoic acid, ethyl ester	0	0	0	0	0	0	0	0	0	0	0	191806	0	0	0	0
Propanoic acid, butyl ester	0	0	0	0	0	0	0	0	0	0	0	52282.7	0	0	0	0
1-Butanol, 2-methyl-, acetate	0	0	0	0	0	0	0	0	0	0	0	68740.7	0	0	219078	0
Propanoic acid, 2-methyl-, ethyl ester	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Octanoic acid, ethyl ester	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ethyl Acetate	14642	203436	9476.67	16169.7	17747.3	14963.3	212771	10867.3	59187.7	81392.7	109332	745307	9139.33	7612	7061	7157
Acetic acid, butyl ester	164491	37120.7	582965	120285	143300	126924	0	1173813	283940	157658	0	161244	124699	92614.3	105444	114122
Acetone	647276	3178832	1398009	1168944	1042082	2319850	1496200	7084425	1840954	1287319	2735618	1438585	8617485	1.6E+07	2.3E+07	8115582
2-Butanone	189126	911262	347992	459441	421346	396215	341801	3615428	341298	601776	875425	310760	4223548	2E+07	1.8E+07	2599510
2,3-Butanedione	160003	9060	7066.67	3751109	4301353	3894575	1.4E+07	1893017	725012	4225038	2.7E+07	256186	2909988	326542	1064820	335118
2,3-Pentanedione	0	0	0	712904	389277	1450296	1629613	0	0	455398	2568765	0	0	2067016	2517785	0
Acetoin	636108	3825.33	3585.67	9394899	5130574	1.3E+07	1.7E+07	1.7E+07	1682368	9502456	3.4E+07	709443	1.9E+07	558493	1277835	597110
2-Hexanone	50885.7	64388	28856.3	201900	128728	260249	349689	139546	94084	159296	167626	89968.7	196407	1393019	1652708	78363.3
2-Heptanone	1.8E+07	2.6E+07	2E+07	3.1E+07	2.5E+07	3.8E+07	3.5E+07	2.7E+07	2.4E+07	3.2E+07	3.7E+07	2.4E+07	2.5E+07	3E+07	2.3E+07	1.7E+07
2-Octanone	294127	279495	249611	530254	291402	829445	0	411413	238777	309857	516845	129745	363746	874645	904891	198660
2-Nonanone	9252190	2.7E+07	1.1E+07	1.3E+07	9245829	1.7E+07	1.5E+07	1.2E+07	8610692	1.1E+07	1.2E+07	1.1E+07	1E+07	1.1E+07	1E+07	8573459
2-Decanone	115584	85186.3	84706.3	150989	32406.3	200564	205150	0	79110.3	125312	169541	0	0	227009	284315	64412.3
2-Undecanone	3371906	2583899	2888098	3487594	3274411	4775508	4786718	3738873	2415134	3629677	3928355	3814021	3231035	3524542	3084261	2651821
Pyrazine, 2-methoxy-3-(1-methylpropyl)-	158045	0	3944755	59098.7	713666	780718	412338	0	0	0	0	0	0	0	0	0
Pyrazine, 2,5-dimethyl-	7337	499210	581583	0	139591	0	30277	409371	987738	0	434311	15475	970263	1.8E+08	1.3E+08	124179
Pyrazine, methyl-	0	0	0	0	0	0	0	0	370847	0	0	0	0	4089953	2537399	0
Pyrazine, 2,3-dimethyl-	0	0	0	0	0	0	0	0	0	0	0	0	0	105817	179120	0
Pyrazine, trimethyl-	0	0	0	0	0	0	0	0	0	0	66101.7	0	0	1.1E+07	1.9E+07	0
Pyrazine, 2,6-dimethyl-2-Methyl-3-isopropylpyrazine	0	0	0	0	0	0	0	0	0	0	0	15475	0	0	0	0
Pyrazine, 3-ethyl-2,5-dimethyl-	0	0	0	0	0	0	0	0	0	0	0	0	0	507970	511567	0
Pyrazine, 2-ethyl-3,5-dimethyl-	0	0	0	0	0	0	0	0	0	0	0	0	0	954347	1957111	0
Pyrazine, 2,5-dimethyl-3-propyl-	0	0	0	0	0	0	0	0	0	0	0	0	0	49403.3	44401.7	0
Pyrazine, 3,5-dimethyl-2-propyl-	0	0	0	0	0	0	0	0	0	0	0	0	0	34853	44401.7	0
Pyrazine, 2-ethyl-5-methyl-	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Disulfide, dimethyl	118790	97715.3	71901.7	20299	30390	15586.3	29841.7	1741602	536957	621992	531181	347381	1794154	4453774	4059120	959064
Dimethyl trisulfide	0	0	0	0	0	0	0	841596	0	0	0	0	634109	236999	178317	221224
1-Propanol, 3-(methylthio)-	0	0	0	0	0	0	0	0	0	0	0	222941	0	0	0	0

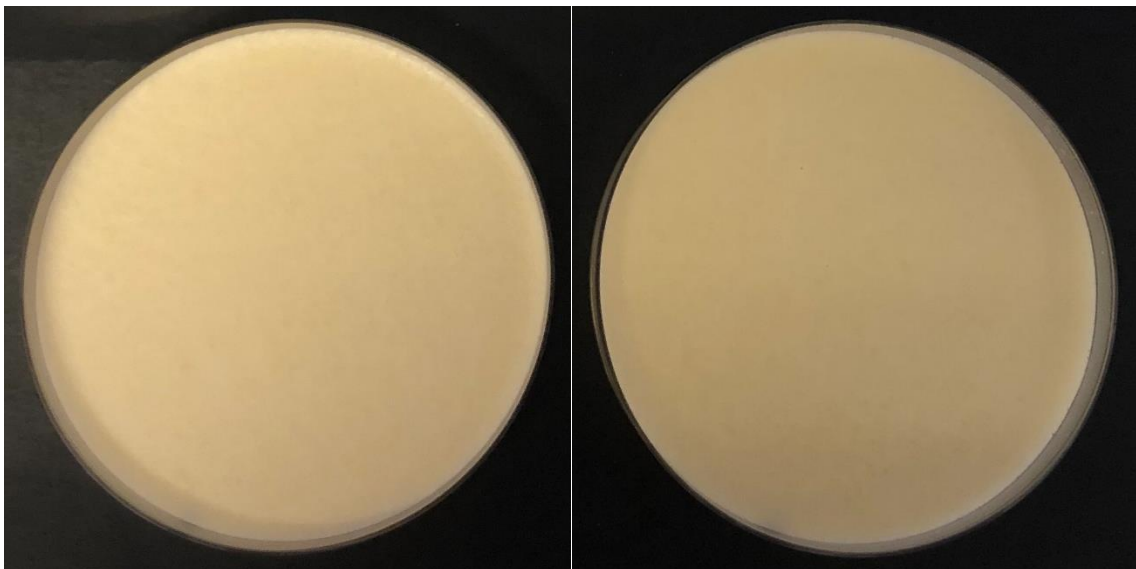
Additional File 1. Photos of biofilms formed by the 43 strains, individually, on a cheese-agar model, after 21 days of ripening at 15°C.

Halomonas



Control

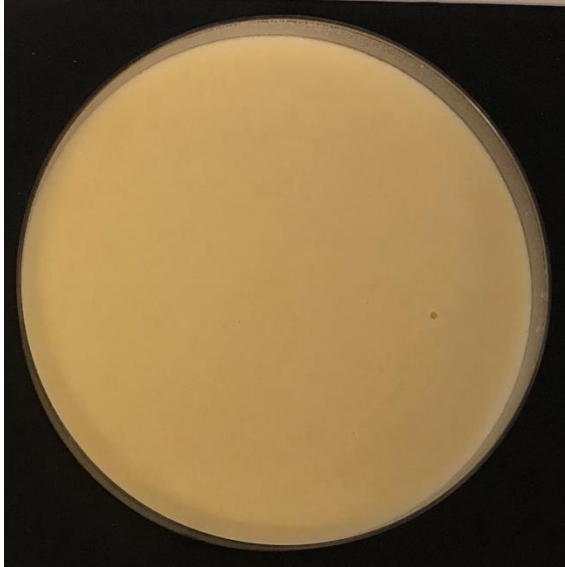
1M45



FME63

FME16

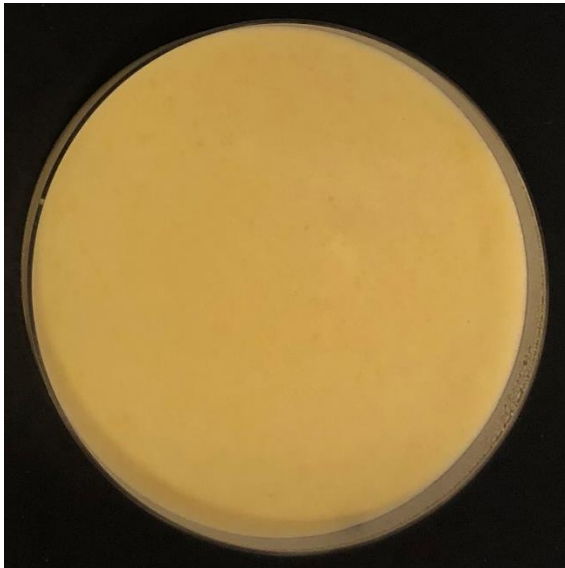
Halomonas



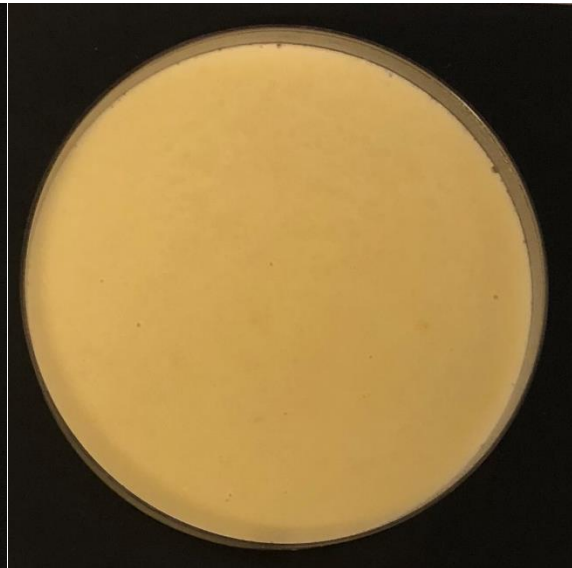
JB380



FME20



FME66

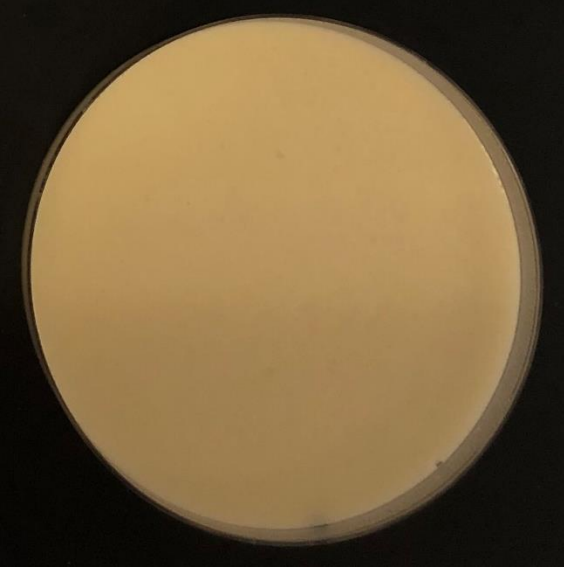


DSM 21076

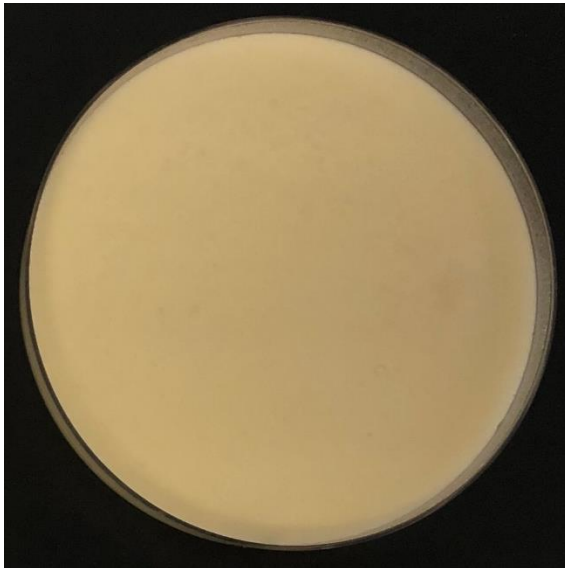
Halomonas



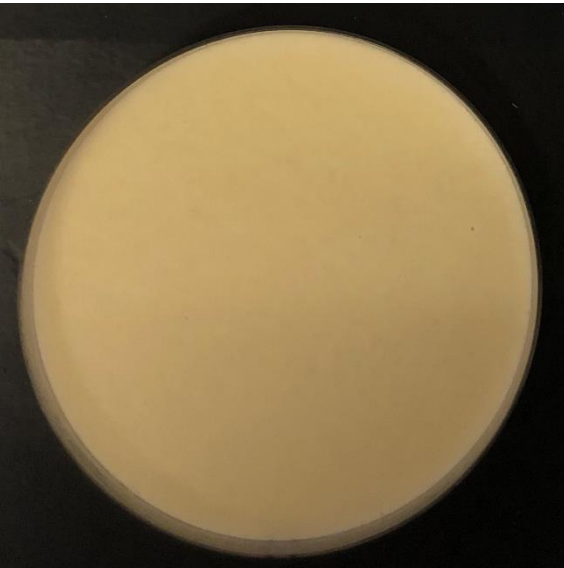
FME1



FME64

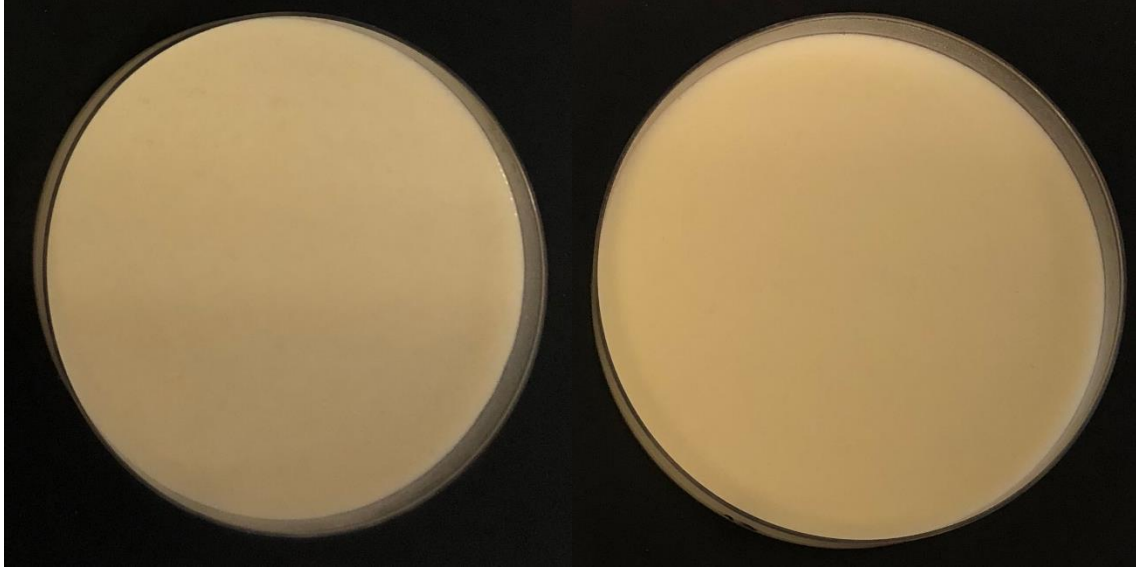


FME65



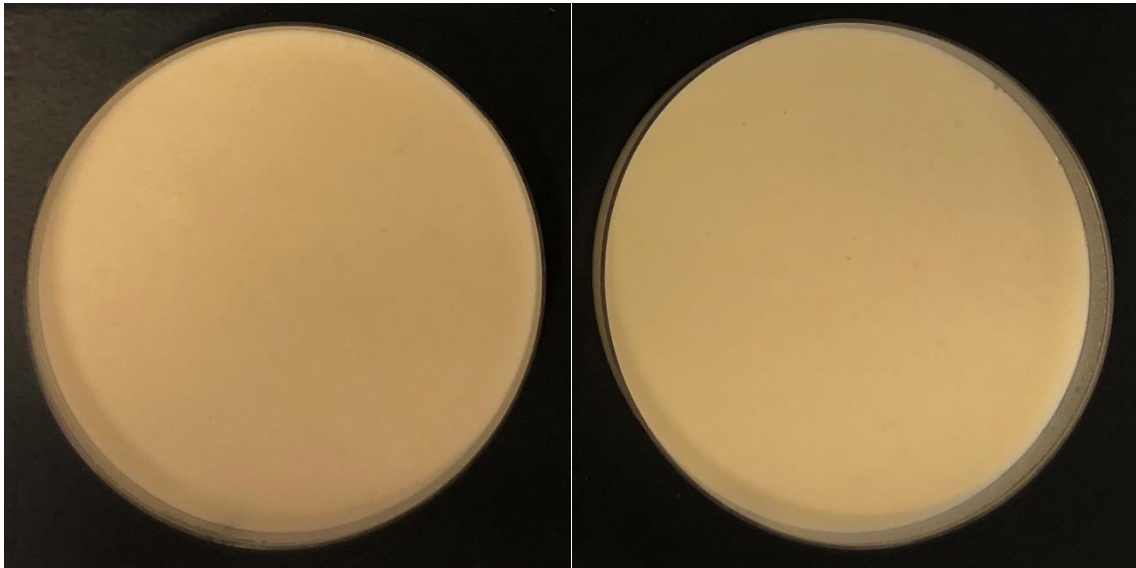
3F2F

Halomonas



3D7M

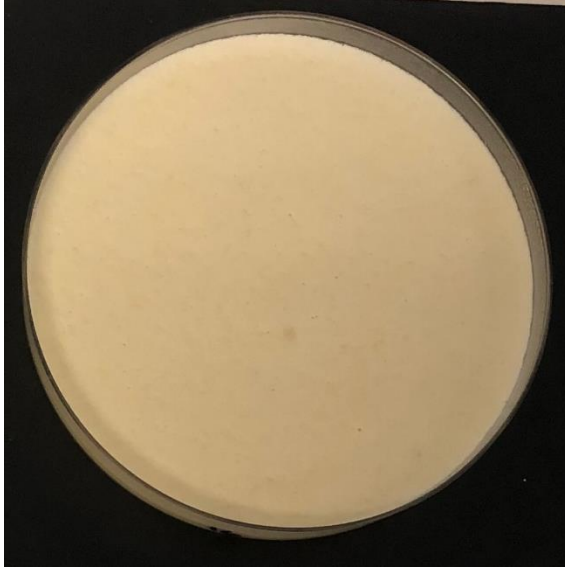
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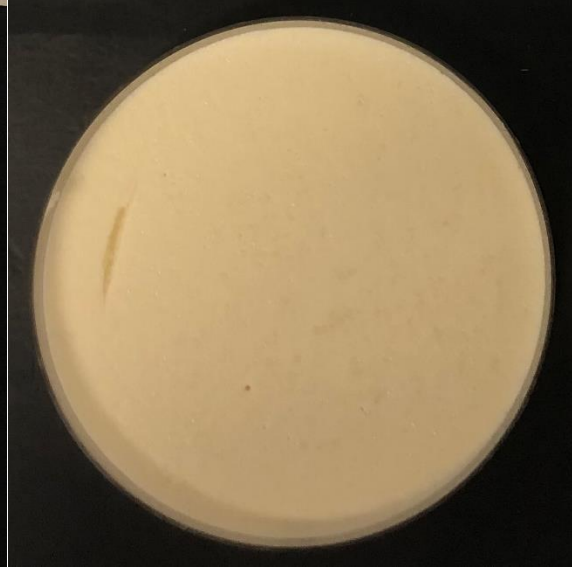
DSM 16354

DSM 4743

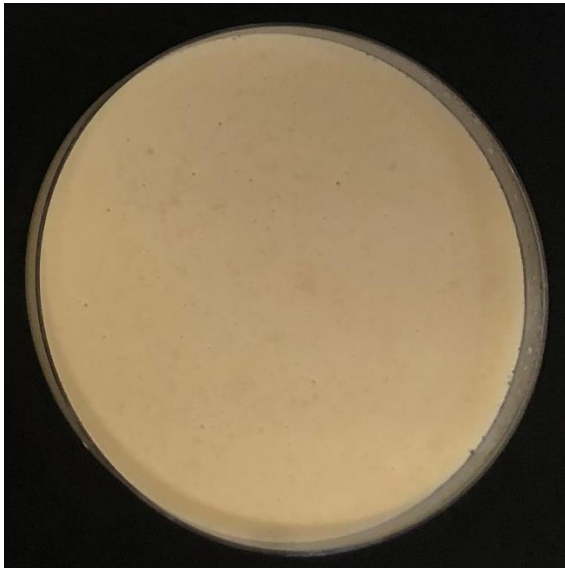
Psychrobacter



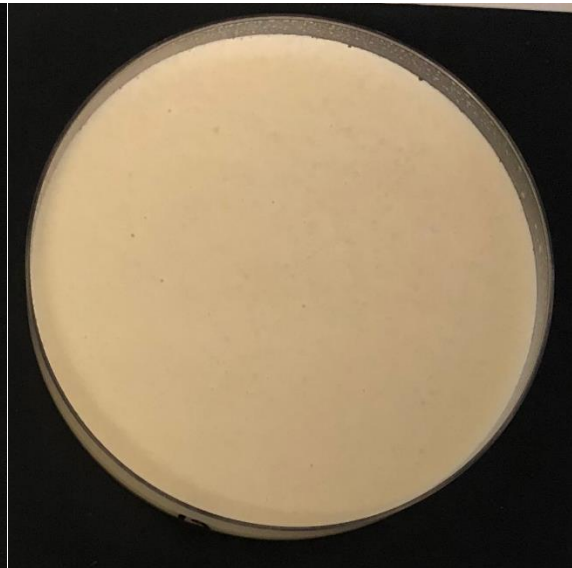
PG1



FME2



FME60



DSM 102806

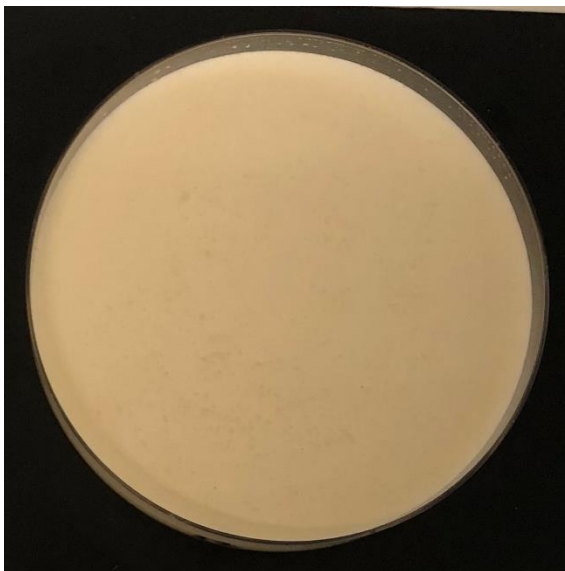
Psychrobacter



DSM 7229



DSM 16327

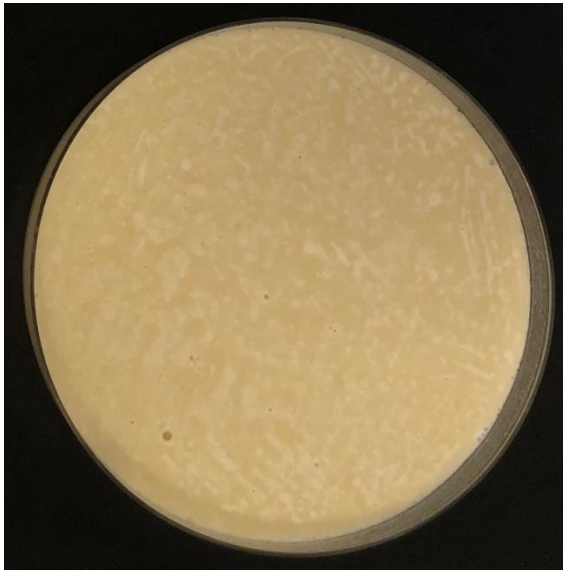


FME6

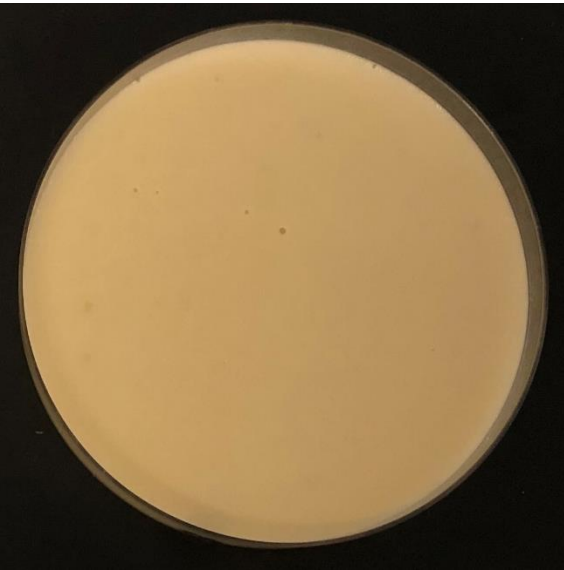


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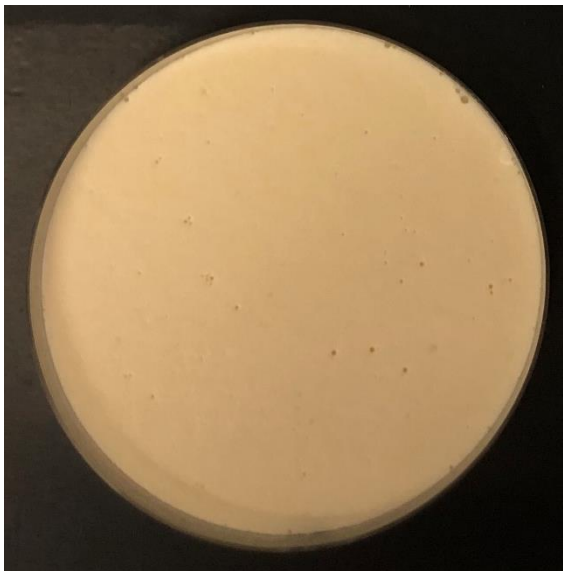
Psychrobacter



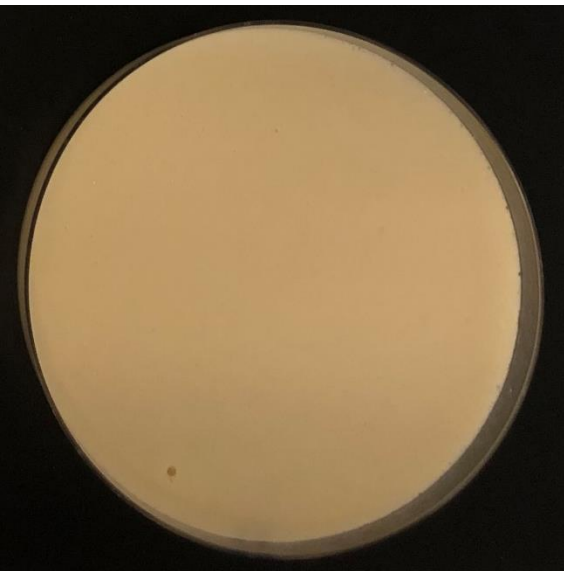
FME13



ER15174BHI

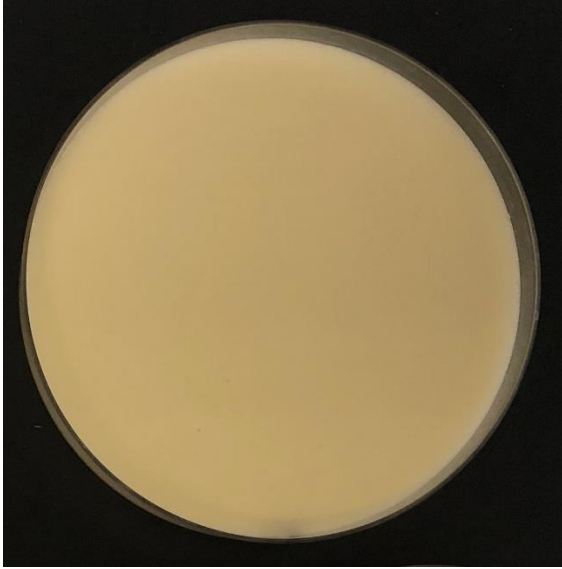


DSM 16329



H5

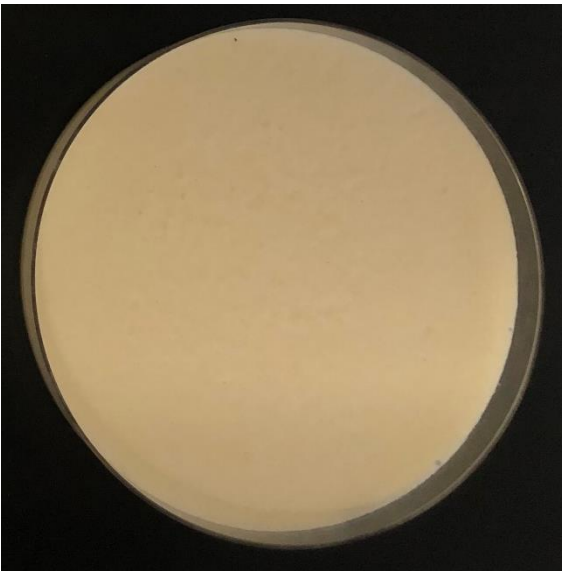
Psychrobacter



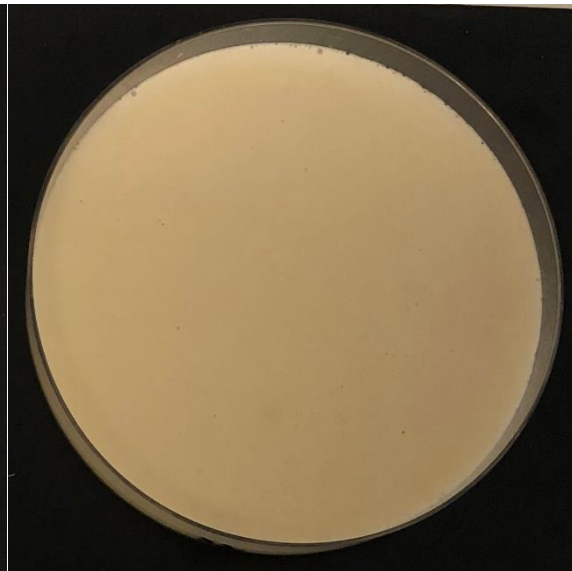
DSM 14664



1439

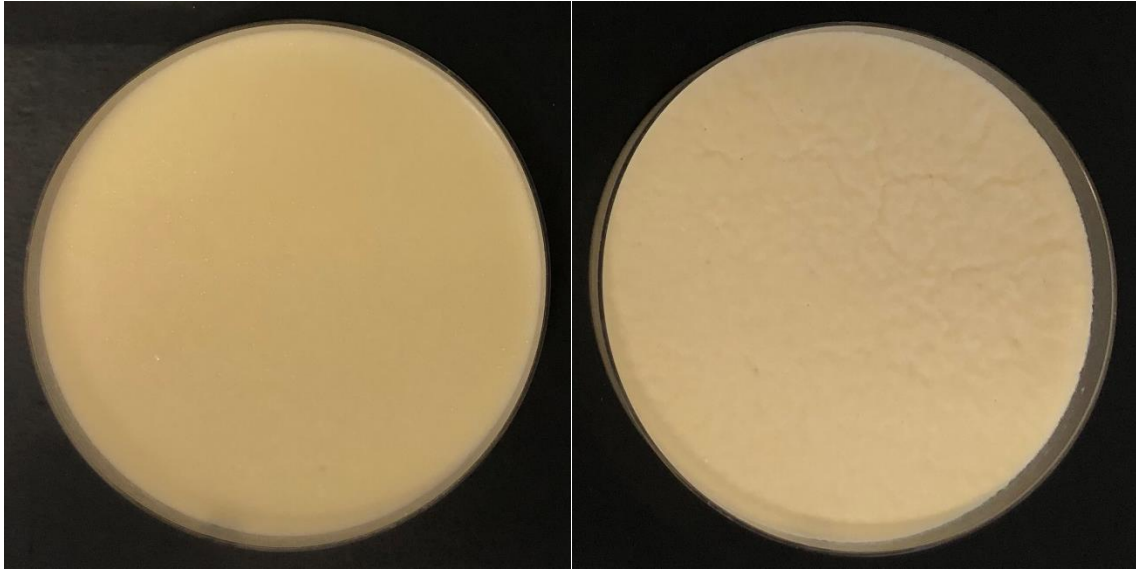


DSM 16330



91

Psychrobacter



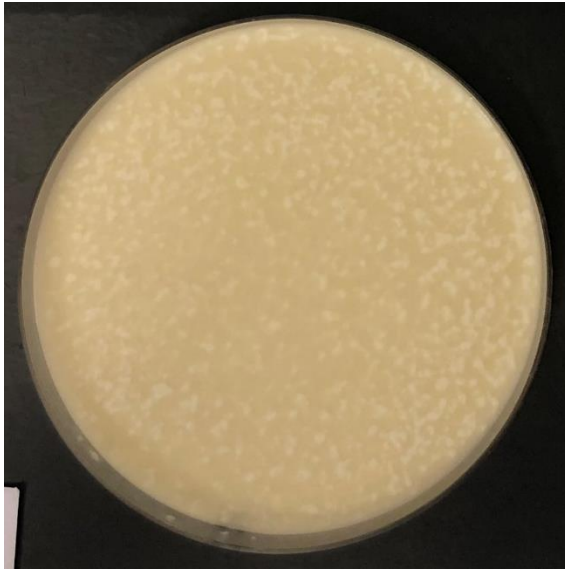
DSM 23510

DSM 16065

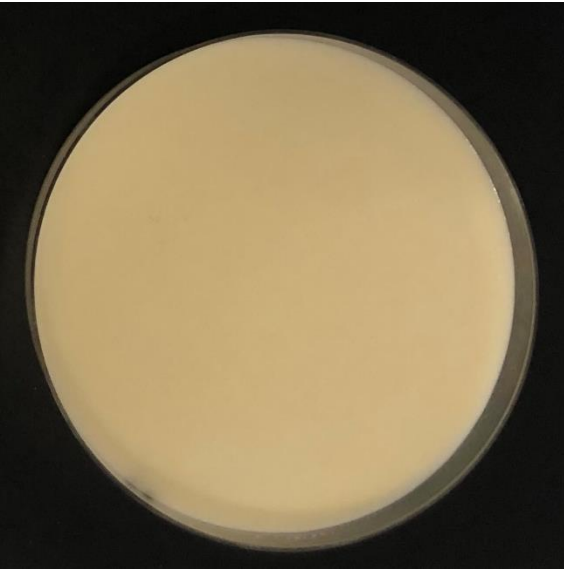


JB385

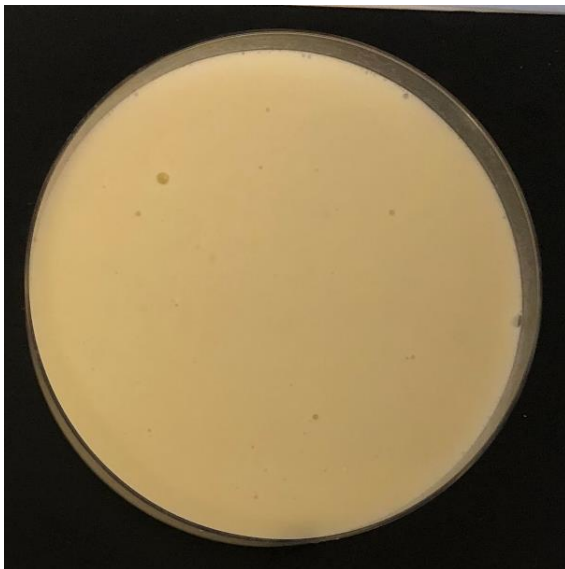
Pseudoalteromonas



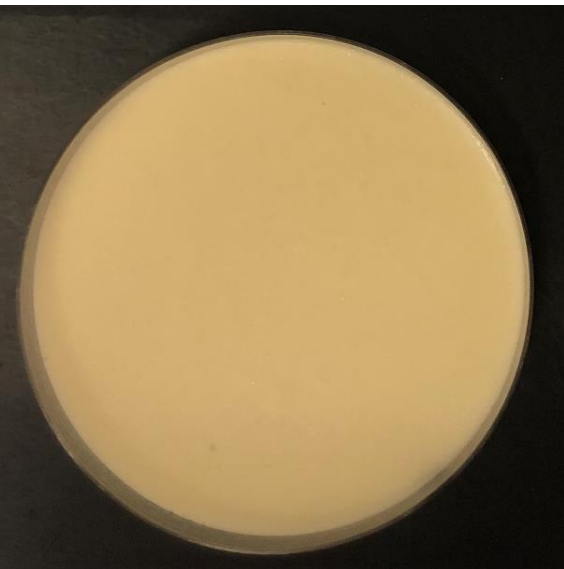
FME14



DSM 14232

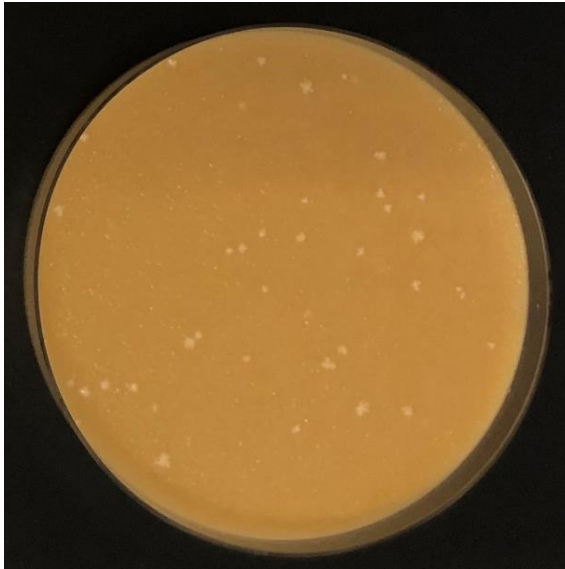


DSM 6060

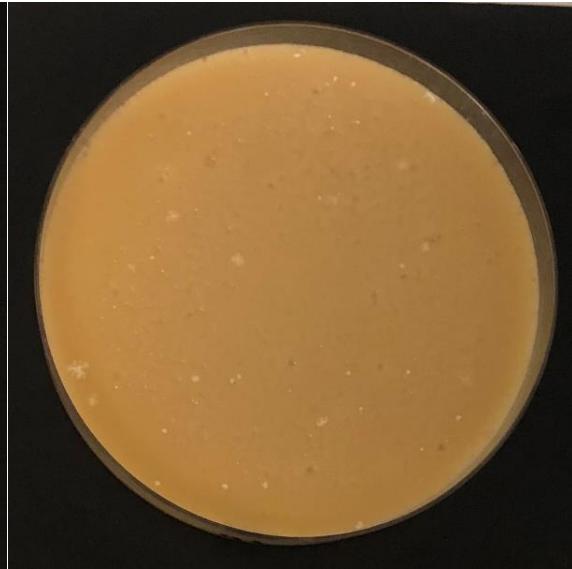


JB197

Pseudoalteromonas



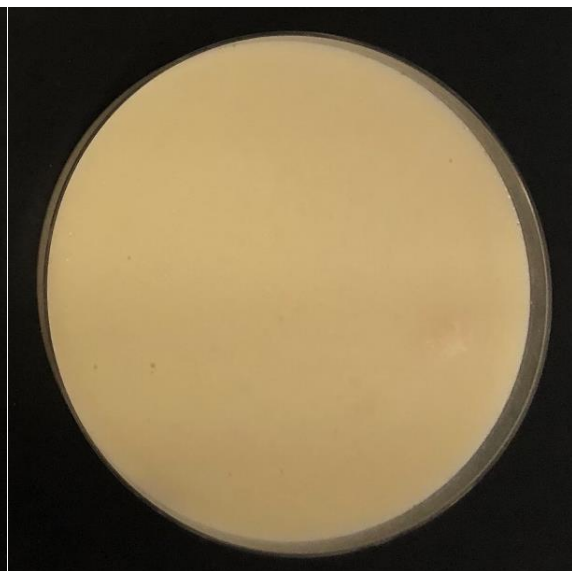
FME68



FME53

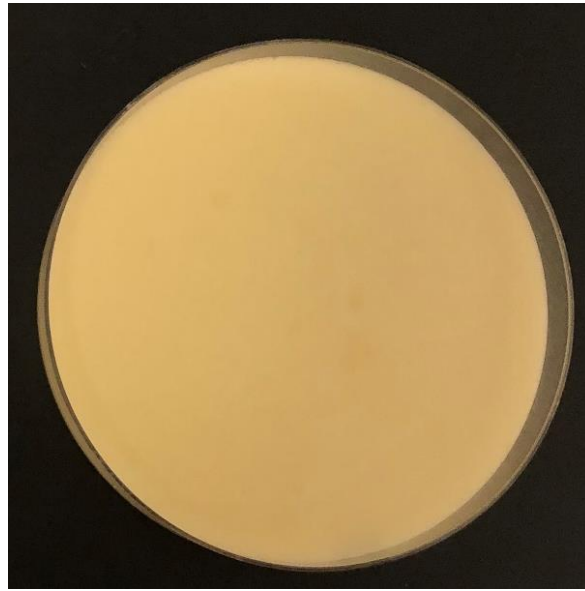


MIP2626



DSM 14402

Pseudoalteromonas



FME67

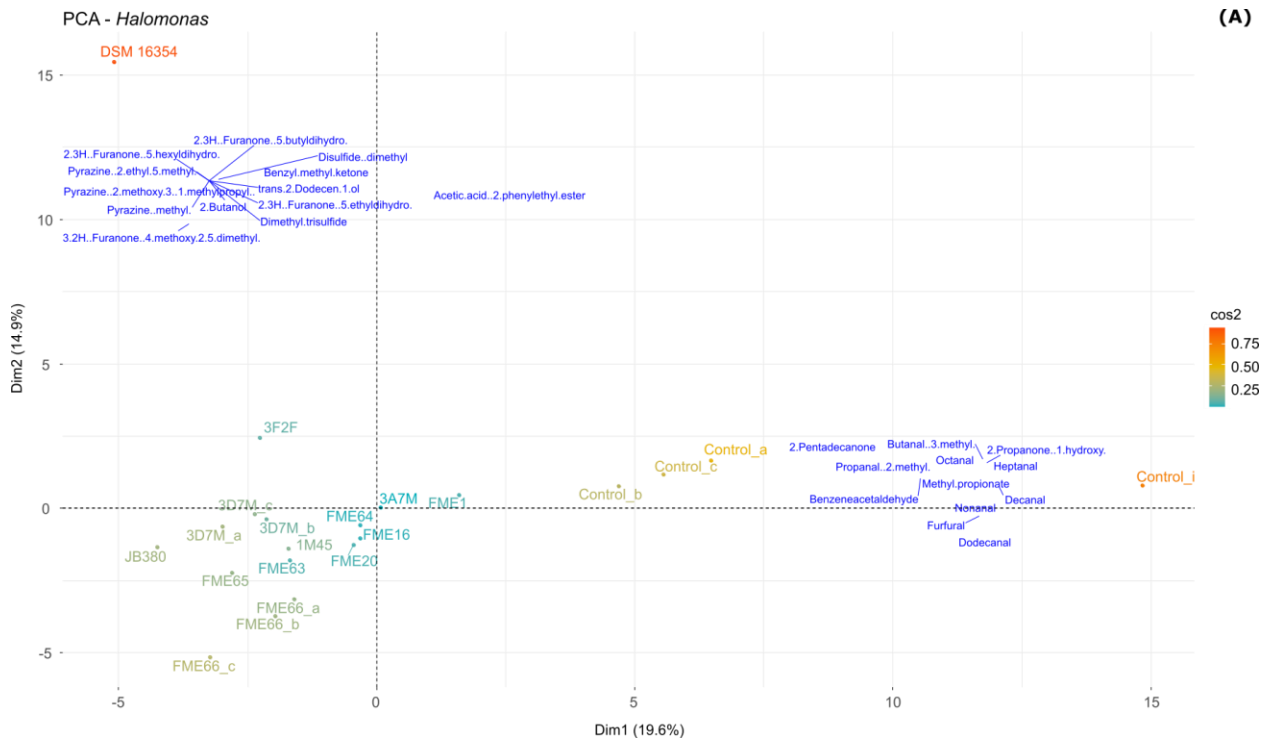


Fig. S1. PCA showing the main volatile compounds produced by *Halomonas* (A) strain.

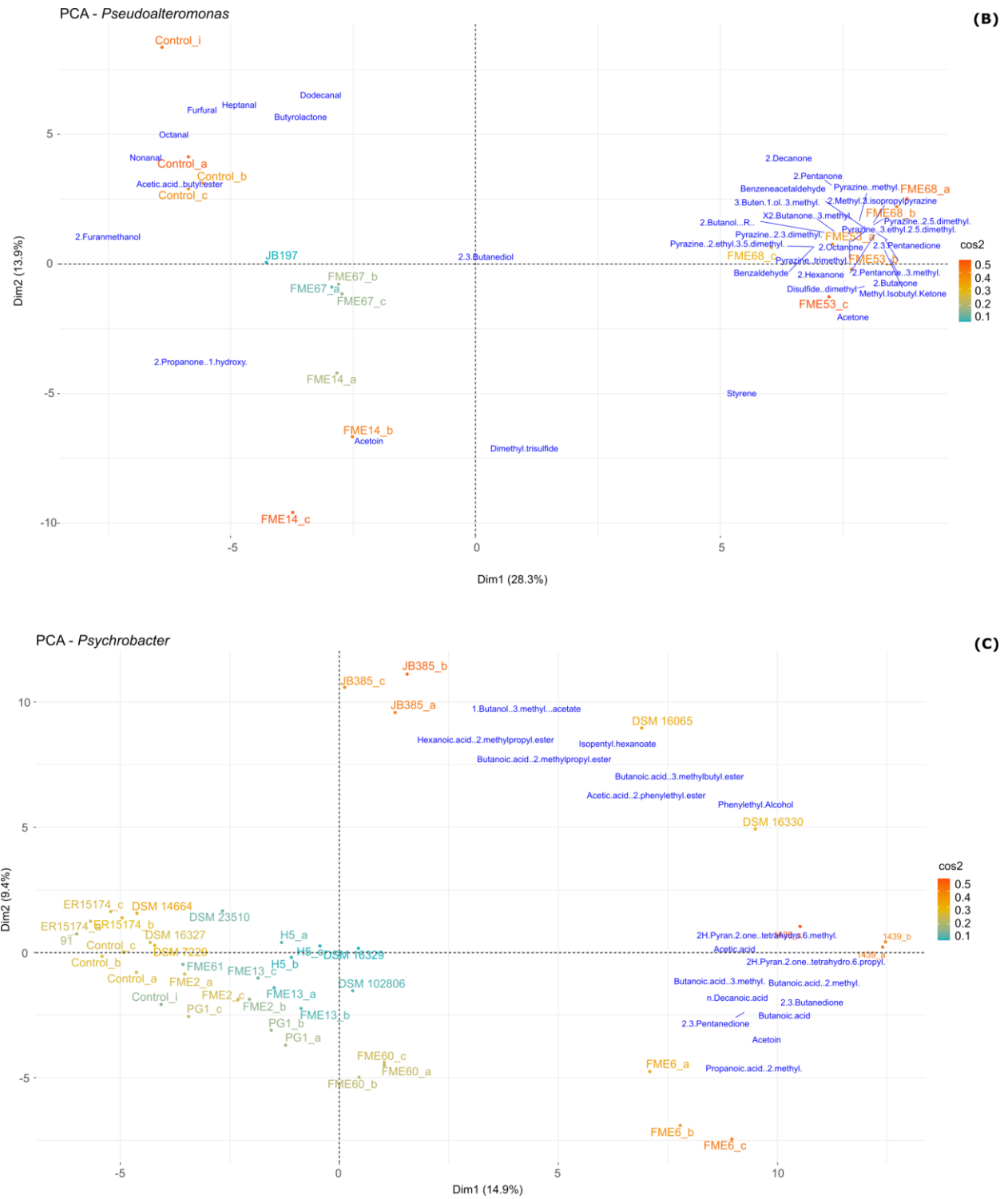


Fig. S1. PCA showing the main volatile compounds produced by *Pseudoalteromonas* (B) and *Psychrobacter* (C) strains.

6 References

- [1]. Irlinger F, Layec S, Hélinck S, Dugat-Bony E (2015) Cheese rind microbial communities: diversity, composition and origin. *Fems Microbiol Lett* 362 (2):1-11. doi:[10.1093/femsle/fnu015](https://doi.org/10.1093/femsle/fnu015).
- [2]. Delbes C, Monnet C, Irlinger F (2015) Des communautés microbiennes au service de la qualité des fromages : diversité et dynamique adaptative et fonctionnelle des populations endogènes etensemencées.
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4.3. Conclusion

Dans cet article, nous avons évalué le potentiel fonctionnel de 43 souches halophiles Gram-négatifs, isolées du fromage et provenant de différents habitats, sur un modèle de fromage. Les résultats indiquent que la plupart des souches, soit alimentaires soit environnementales, ont pu se développer dans le caillé-agar. De plus, nous avons constaté que les rôles potentiels des souches différaient considérablement dans la production de composés volatils, variations de pH et aspects visuels à la surface des modèles. De manière générale, les souches fromagères d'*Halomonas* n'avaient pas d'odeurs perceptibles, mais plusieurs d'entre elles augmentaient le pH du fromage-agar. Deux souches de *Pse. nigrifaciens* (FME68 et FME53) présentaient un potentiel aromatique élevé, mis en évidence par l'analyse sensorielle et probablement associé à la production d'une variété de composés volatils, tels que les pyrazines, cétones et composés soufrés. Ces deux souches ont aussi révélé une coloration brune à la surface des modèles, qui peut être liée à la production de pyrazines. Deux souches de *Psychrobacter* (*Psy. casei* FME6 et *Psy. namhaensis* 1439) se distinguent également des autres souches, en produisant une variété d'acides carboxyliques et en acidifiant le pH dans le caillé-agar. Ces souches ont produit du diacétyl et de l'acétone, des composés volatils qui génèrent une odeur beurrée, décrite par les évaluateurs lors de l'analyse *sniffing*. De plus, quelques souches de *Psychrobacter*, notamment les souches DSM 16065 et JB385, ont formé un biofilm blanc accompagné de textures froissées à la surface des modèles de fromage.

En conclusion, cette étude a mis en évidence le rôle de plusieurs protéobactéries qui font partie de la flore dominante dans plusieurs fromages à croûte lavée et dont l'influence au niveau des arômes, textures et couleurs, peut intéresser la technologie fromagère.

CHAPITRE III – DISCUSSION ET PERSPECTIVES

L'objectif de cette thèse consistait à la fois, à étudier les espèces halophiles, présentes sur les croûtes de fromages, pour lesquelles les études sont, à ce jour, peu développées, et à mieux comprendre leurs interactions potentielles dans la technologie fromagère. Dans cette perspective, la flore bactérienne tolérante vis-à-vis du sel a d'abord été mise en évidence par des méthodes culturales et métagénomiques, puis, par une connaissance approfondie de ses caractéristiques génotypiques et phénotypiques ainsi que de son rôle potentiel dans les fromages. Les informations obtenues ici, au-delà de leur valeur scientifique, présentent aussi un intérêt technologique pour les fromagers et culturel pour les consommateurs de ces anciens produits fermentés.

1. Les halophiles: une avancée dans la compréhension des écosystèmes fromagers

Jusqu'à présent, les halophiles ont été étudiés principalement au sein des milieux environnementaux (mer, lacs, sols, etc). Cependant, des études récentes révèlent, par des méthodes indépendantes de la culture, que certaines protéobactéries tolérantes au sel sont présentes également dans les aliments. Nous avons ainsi constaté, en début de thèse, un déficit d'isolats halophiles alimentaires dans les centres de ressources biologiques, nationaux et internationaux. Cela peut être associé au manque d'intérêt des chercheurs pour isoler ces bactéries dans la nourriture, considérées comme éléments contaminants, contrairement à la grande faveur manifestée pour les bactéries lactiques. Une deuxième raison pourrait résider dans l'utilisation de milieux de culture inappropriés pour les cultiver (sans ajout de sel, par exemple). En effet, lors de ce travail, nous avons été amenés à développer des stratégies destinées à isoler des bactéries halophiles à partir de croûtes de fromage.

Bilan des bactéries halophiles dans les fromages

Pour établir le bilan de la diversité des bactéries halophiles dans les croûtes de fromage, nous avons compilé tous les génomes des genres *Halomonas*, *Marinomonas*, *Pseudoalteromonas*, *Psychrobacter* et *Vibrio*, isolés dans l'ensemble des études portées à notre connaissance concernant l'écosystème fromager [3, 4, 228, 229], ainsi que des MAGs correspondant à des

espèces non encore isolées [47]. Au total, 43 génomes halophiles fromagers ont été rassemblés et un arbre phylogénomique a été construit (Fig. 9). Parmi eux, 19 ont été isolés et séquencés dans ce Projet (Publication 1 et 2, génomes marqués par des étoiles noires) et 10, provenant d’une étude antérieure de notre équipe (étoiles bleues, [4]).

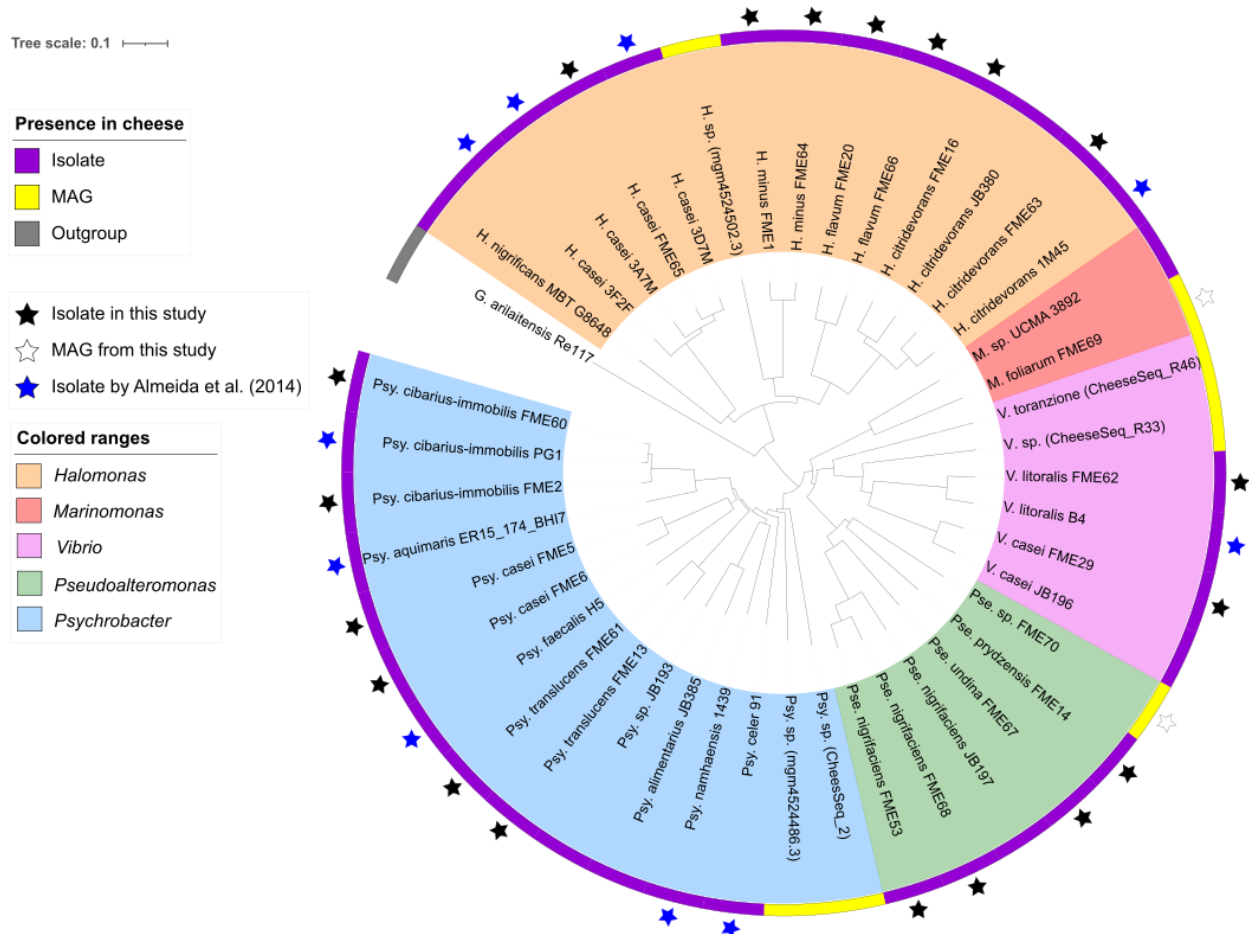


Figure 9. Les 43 génomes halophiles, signalés dans l'écosystème fromager.

Pour définir les différents clades au sein de cette diversité, des analyses des ANIs ont été réalisées avec l'ensemble des données génomiques (données non présentées). Parmi les résultats, six espèces fromagères peuvent être identifiées en tant que genre *Halomonas*. Quatre d'entre elles ont été assignées dans ce travail de thèse (Publication 3), et attribuées à : *H. citridevorans*, *H. minus*, *H. casei* et *H. flavum*. Les deux groupes restants appartiennent à l'espèce *H. nigrificiens* (obtenue à partir d'un fromage à caillé acide appelé Quargel [229]) et à une espèce non encore

isolée (MAG), obtenue à partir d'un fromage à croûte lavée, fabriqué en Rhône-Alpes, France [47].

Concernant les *Marinomonas*, ils sont moins fréquemment signalés dans l'alimentation et ne sont représentés que par deux espèces dans les fromages (**Fig. 9**). À notre connaissance, un seul génome a été séquencé à partir d'une souche du fromage pour ce genre (*Marinomonas sp.* UCMA 3802, isolé du Livarot [228]), dont l'assignation à une espèce reste à établir. La deuxième espèce de *Marinomonas* est actuellement composée d'un seul représentant alimentaire, correspondant au génome reconstitué dans cette étude (**Publication 2**, souche FME69), qui peut être attribué à l'espèce *Marinomonas foliarum* en raison de sa proximité avec la souche type respective CECT 7731^T (ANI=97%).

Dans le groupe des *Pseudoalteromonas*, trois espèces, parmi les quatre détectées, ont des représentants isolés. L'espèce *Pse. nigrifaciens* est celle qui est le plus souvent détectée dans les croûtes de fromage, mais nous avons aussi isolé des souches représentatives des espèces *Pse. pyrdzensis* et *Pse. undina*. La dernière espèce de *Pseudoalteromonas*, représentée ici par un MAG (souche FME70) ne correspond à aucun représentant isolé, mais son génome a été reconstitué dans notre étude (**Publication 2**). Walsh *et al.* [47] ont aussi obtenu un MAG de cette même espèce. Il est possible que ces MAGs proviennent d'un fromage avec la même appellation, puisque les deux MAGs sont originaires de croûtes de fromage au lait de vache non pasteurisé d'Auvergne, France.

Nous avons observé une très grande diversité d'espèces dans les fromages au sein du genre *Psychrobacter*, constitué de onze clades (**Fig. 9**). Deux souches obtenues dans ce travail, FME2 et FME60, partagent des ANI avec un pourcentage très proche, entre 95-96%, avec *Psy. cibarius* et *Psy. immobilis*, indiquant qu'elles sont soit au seuil de résolution d'une nouvelle espèce, soit qu'elles pourraient être assignées à l'une de ces deux espèces. Une analyse plus approfondie, incluant des caractéristiques phénotypiques, génomiques et chimiotaxonomiques, doit être effectuée pour vérifier leur taxonomie. En outre, deux espèces détectées dans les fromages ont été attribuées au cours de ce travail, à : *Psy. casei* et *Psy. translucens* (**Publication 4**). D'autres espèces de *Psychrobacter*, décrites notamment par Almeida *et al.* [4], sont également identifiées dans les croûtes de fromage, telles que *Psy. alimentarius*, *Psy. aquimaris*, *Psy. celer*, *Psy. faecalis* et *Psy. namhaensis*. Cependant, trois groupes restent encore sans assignation d'espèce dans ce genre, dont deux correspondent aux MAGs récemment reconstitués par Walsh *et al.* [47], à partir de métagénomés de fromages irlandais, et le troisième, identifié comme la souche JB193, qui a été

isolée par Wolfe *et al.* [3] (fromage non mentionné) dont le représentant le plus proche est *Psy. alimentarius* (ANI=90 %).

Nous avons également mis en évidence la présence de plusieurs espèces de *Vibrio* dans les fromages, dont quatre clades distincts ont été identifiés. Les deux groupes les plus répertoriés dans les fromages correspondent à *V. casei* et *V. litoralis* et disposent de représentants isolés, y compris dans cette étude (FME29 et FME62, respectivement). Les deux autres espèces ne comportent aucun isolat alimentaire, seulement des MAGs récupérés à partir de fromages irlandais [47]: l'un d'eux peut être attribué à *Vibrio toranzoniae* (ANI>97,7% avec la souche type CECT 7225^T), tandis que l'autre espèce reste encore sans assignation.

En conclusion, ce bilan nous a permis d'identifier une grande diversité de génomes halophiles fromagers appartenant à 27 espèces distinctes (6 *Halomonas*, 2 *Marinomonas*, 4 *Pseudoalteromonas*, 11 *Psychrobacter* et 4 *Vibrio*). Les isolats disponibles forment un ensemble de références d'espèces de bactéries alimentaires, qui mériterait d'être exploré pour leur potentiel métabolique, leur rôle technologique et leurs applications biotechnologiques. Cependant, sept espèces n'ont pas encore été cultivées et ne sont présentées que sous forme de MAGs. Afin de guider l'isolement de ces espèces et de connaître la représentativité des autres espèces pour évaluer leurs potentiels, il est utile de connaître dans quels types de fromages il faut aller les chercher. Ainsi, dans la section suivante, nous discuterons de la fréquence et de l'abondance de ces 27 espèces halophiles dans les croûtes de fromage.

Les protéobactéries comme population bactérienne dominante dans les croûtes de fromages

Afin de fournir une vision complète des bactéries halophiles présentes dans les croûtes de fromage, un génome de chaque espèce a été utilisé comme référence pour les détecter et les quantifier au niveau des données métagénomiques. Pour cela, nous avons sélectionné un ensemble de 114 échantillons correspondant à des croûtes de fromage de différents types, dont 49 provenant de notre étude ([Publication 1](#)) et 65 d'études précédentes [3, 4, 47, 230] (**Tableau S1**, [Annexe 3](#)). Parmi les croûtes de fromage analysées, seules 13 ne présentent pas de niveaux détectables (pourcentage au moins égal à 0,1%), pour les espèces halophiles des genres *Halomonas*, *Marinomonas*, *Pseudoalteromonas*, *Psychrobacter* et *Vibrio*. Celles-ci correspondent à différents types de croûtes, mais plus particulièrement, les naturelles et fleurées, avec pâtes molles à dures, incluant un fromage à pâte persillée. Les abondances relatives des *reads*, correspondantes aux 27

génomomes de référence cartographiés sur les 101 métagénomomes restants, sont présentées dans la [Fig. 10](#). En particulier, 32 échantillons à pâte molle et croûte lavée, présentent des abondances relatives supérieures à 10 % des *reads* de ces bactéries, ce qui montre leur importance dans ces produits. Il est intéressant de noter que ces espèces se retrouvent non seulement dans les fromages français, mais aussi dans les fromages irlandais, anglais et nord-américains.

Bien que différentes méthodes de regroupement aient été testées et une tendance des bactéries halophiles a été observée dans les pâtes molles et croûtes lavées, la classification par mode technologique et par type de croûte de fromage n'a pas permis de faire ressortir des corrélations claires. De plus, certaines études ne mentionnent pas toutes les caractéristiques des métagénomomes (marquées comme « unknown »), et la définition des catégories est alors délicate. Cette incertitude peut concerner, par exemple, les fromages qui renferment des levures dans leur croûte (que l'on pourrait définir comme une croûte fleurie), mais qui ont aussi été frottés avec de l'eau salée (croûte lavée) (Cahier de charges du Saint Nectaire, 2017). Ainsi, cette classification peut rester très subjective selon la personne qui la catégorise, et, également, présenter des tendances différentes, selon le type de pâte. De plus, nous ne disposons pas d'information sur le temps d'affinage de ces fromages, ce qui pourrait être une donnée intéressante pour déduire la cinétique d'implantation des halophiles à la surface des fromages, depuis le début de la production jusqu'à leur sortie des caves d'affinage. Des recherches futures peuvent être menées pour étudier l'évolution de ces microorganismes tout au long de la production, ainsi que pour identifier quels matériaux et ingrédients, utilisés dans la fromagerie, sont les vecteurs des bactéries halophiles.

Parmi les espèces analysées, les dominantes (au moins cinq échantillons >10%) et fréquentes (au moins 30 échantillons > 0,1%), dans les croûtes de fromages, sont : *Psy. cibarius-immobilis*, *Psy. translucens*, *H. citridevorans* e *Pse. nigrifaciens*, dont certaines pourraient interagir, car elles sont détectées fréquemment ensemble et potentiellement associées. Concernant les espèces non encore isolées, à l'exception de *Psychrobacter sp.* (CS_2), elles apparaissent fréquemment et parfois en abondance, en particulier les espèces d'*Halomonas sp.* (mgm4524502.3), *Psychrobacter sp.* (mgm4524486.3) et *Vibrio sp.* (CS_R33). Elles ont été détectées principalement dans les croûtes lavées ou naturelles des fromages à pâte molle français ou irlandais. Un travail plus approfondi dans ce sens devrait contribuer à reconstituer des écosystèmes fromagers afin d'étudier le rôle de cette flore tolérante au sel.

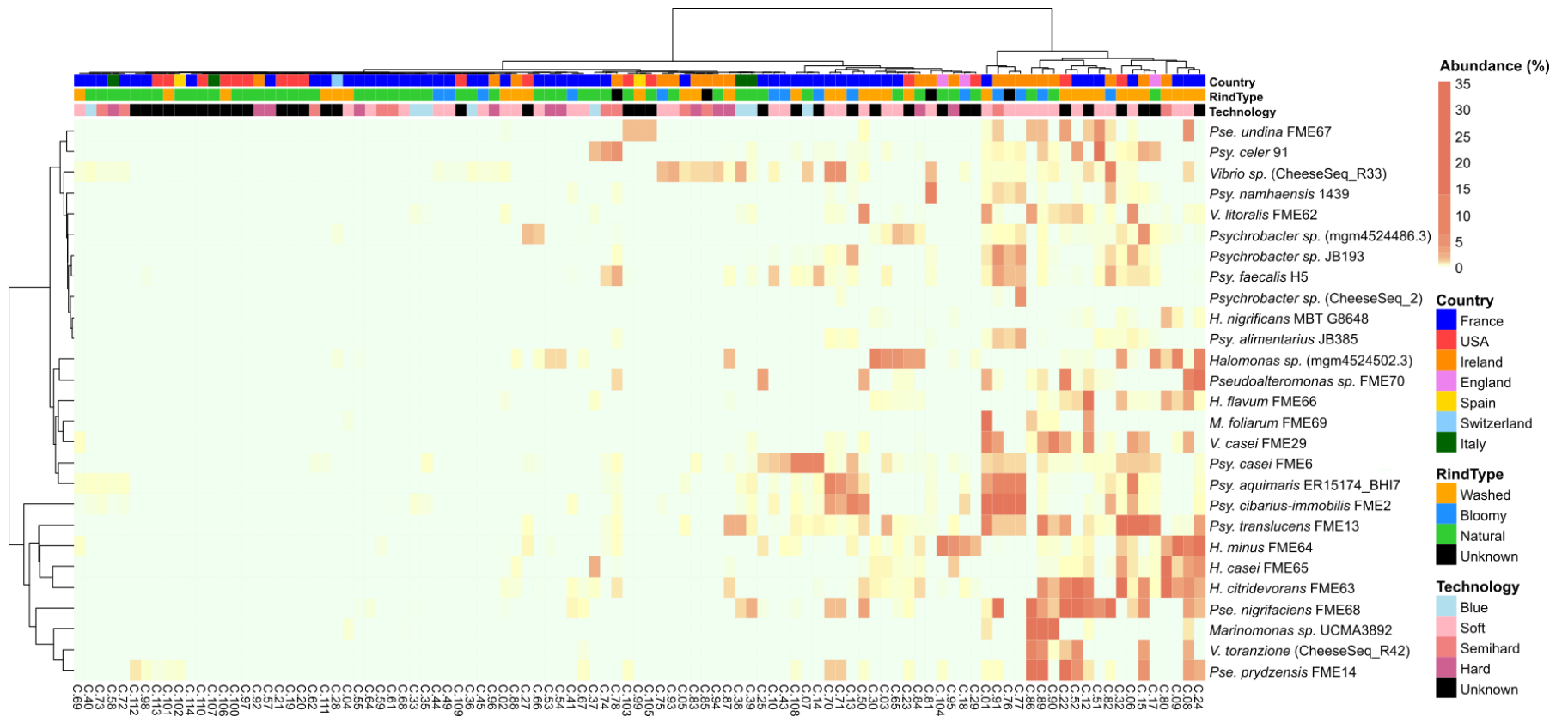


Figure 10. Heatmap illustrant l'abondance relative (%) de 27 espèces halophiles dans 101 croûtes de fromage.

Les résultats obtenus peuvent également guider la culture des espèces non encore isolées, en ciblant les fromages dont elles sont dominantes. Ensuite, nous avons proposé, dans cette étude, une stratégie pour faciliter et cibler l'isolement de représentants d'espèces détectés par la présence de leur séquence, en concevant des sondes spécifiques pour les révéler rapidement à partir de clones isolés sur boîte. Cette stratégie s'est montrée efficace dans de nombreux cas. Cependant, nous n'avons pas réussi à cultiver deux espèces (*Marinomonas foliarum* FME69 et *Pseudoalteromonas* sp. FME70), malgré des conditions initiales favorables dont leur forte abondance ([Publication 2](#)). Compte tenu du fait que notre analyse génomique n'a pas révélé de différence notable avec leurs plus proches parentes cultivables, concernant les cellules bactériennes, l'hypothèse qui nous paraît la plus plausible est que celles-ci aient été détruites par la congélation, sachant que les échantillons ont été conservés à une température de -20 °C. Ainsi, nous suggérons, pour les travaux futurs, de tester leur présence dans une série d'échantillons de croûtes de fromages, sans les congeler, et aussi, d'explorer une plus large gamme de milieux de culture. En effet, même si le milieu *Marine Agar* s'est avéré être le plus efficace, lors de notre travail pour isoler les bactéries halophiles, d'autres milieux pourraient être utilisés pour augmenter la probabilité d'isolement. Par exemple, une étude récente sur la flore halophile des saumures de fromage a utilisé deux milieux de culture à forte concentration en sel : (i) GYPB, composé de glucose, extrait de levure, peptone, extrait de bœuf, avec sel et (ii) MGM, composé d'extrait de malt, d'extrait de levure et de sel [100].

2. Peut-on retracer l'évolution des bactéries halophiles dans l'écosystème fromager?

Dans le but de mieux caractériser la diversité des halophiles du fromage appartenant aux genres *Halomonas*, *Marinomonas*, *Pseudoalteromonas*, *Psychrobacter* et *Vibrio*, les génomes de représentants de différentes espèces issues d'autres écosystèmes ont été collectés dans la base de données [NCBI](#). Des arbres phylogénomiques ont été construits, en indiquant leurs habitats (fromage, autres aliments, environnement et contexte humain, figures supplémentaires S1-S5, [Annexe 3](#)). À l'exception de *Psychrobacter*, pour lequel les représentants alimentaires semblent dominer, les autres genres sont majoritairement issus d'écosystèmes naturels.

colonies. En outre, *H. casei* est étroitement apparenté (ANI~95-96%) aux espèces *H. hydrothermalis*, *H. venusta*, *H. alkaliphila* et *H. campaniensis*, toutes, isolées du milieu marin. Pour les autres genres étudiés, la majorité des espèces de fromage sont également représentés dans les habitats naturels ([Fig. 11](#)), à l'exception de *V. casei* et *Psy. casei*. Enfin, il est intéressant de noter que les MAGs dont les représentants restent à isoler (*Halomonas sp.*, *Vibrio sp.*, *Pseudoalteromonas sp.* et *Psychrobacter spp.*) n'ont pas non plus de représentants au niveau d'un génome proche isolé. Il est pertinent de se demander si ces absences révéleraient une difficulté particulière concernant la culture de souches appartenant à ces espèces. Enfin, nous constatons la présence d'espèces isolées au sein d'autres aliments, génétiquement proches des souches fromagères et environnementales. C'est le cas de la *Pse. nigrifaciens* DSM 6063^T, isolée à partir du beurre, et de la *Psy. alimentarius* JG-102^T et *Psy. cibarius* JG -219^T, tous deux isolées du *jeotgal*, un aliment fermenté traditionnel coréen, à base de fruits de mer.

Cette analyse préliminaire indique que les bactéries alimentaires sont peut-être issues de souches appartenant à l'environnement naturel. Pour mieux répondre à cette hypothèse, des analyses comparatives de génomes pourraient être effectuées pour mettre en évidence les gènes présents dans les bactéries du fromage et absents dans les autres milieux, ou vice-versa, et ainsi indiquer les processus d'adaptation potentiels pour coloniser les écosystèmes alimentaires.

3. Quel est le rôle des halophiles dans la technologie fromagère ?

La fréquence et parfois l'abondance de certaines espèces halophiles dans les croûtes de fromage ([Fig. 10](#)) suggèrent que celles-ci pourraient jouer un rôle technologique sous-estimé jusqu'à présent. Cette constatation a motivé une première approche pour tester les impacts technologiques potentiels des souches halophiles dans un modèle fromage-agar. Pour cela, nous avons d'abord rassemblé un certain nombre d'isolats des genres *Halomonas*, *Pseudoalteromonas* et *Psychrobacter*, provenant des écosystèmes alimentaires et naturels, soit un total de 43 souches (Tableau 1, [Publication 5](#)). Les souches issues d'environnement non fromager ont été introduites pour tester en parallèle leurs potentiels. À partir de cette collection, nous avons testé la capacité d'implantation de chaque souche, individuellement, dans un modèle de fromage synthétique, et leur impact sur le pH et la production de composés volatils, après 21 jours d'incubation à 15 °C (en simulant un affinage initial). En général, on observe une bonne capacité de croissance des

souches halophiles dans le modèle fromage-agar, y compris pour les souches environnementales. En ce qui concerne les souches fromagères, on observe que certaines d'entre elles (notamment les souches de *Psy. casei* FME6 et *Psy. namhaensis* 1439) peuvent légèrement acidifier le milieu et produire une série d'acides carboxyliques et de cétones qui pourraient renforcer la qualité aromatique des fromages. De plus, deux souches de *Pseudoalteromonas nigrifaciens* (FME68 et FME53) produisent des pyrazines et des cétones ainsi que des composés soufrés, éléments pouvant contribuer à l'arôme lors de l'affinage du fromage. Ces classes de composés volatils ont déjà été décrites dans l'étude des fromages [231]. Selon la quantité produite ainsi que les préférences des consommateurs en matière de goût, elles peuvent être considérées comme des odeurs déplaisantes ou souhaitables.

Ces recherches préliminaires nous permettent d'envisager des analyses plus approfondies concernant les souches qui ont démontré des potentiels technologiques prononcés. Par ailleurs, est-il possible de considérer que certaines souches halophiles peuvent être inoculées, comme ferments, dans les surfaces de fromage ? Bien que certains auteurs puissent être sceptiques, face à une réponse affirmative à cette question, puisque les espèces étudiées appartiennent au groupe des protéobactéries qui leur sont normalement associées comme contaminants ou éléments d'altération des aliments, certaines souches étudiées ici ont montré une utilisation potentielle dans la technologie fromagère. Toutefois, les perspectives de travail suivantes restent ouvertes à une exploration détaillée :

a) Comprendre l'implantation et le rôle des halophiles dans l'écosystème fromager

Selon le *heatmap* présenté ci-dessus, les bactéries halophiles sont dominantes essentiellement dans les fromages à croûte lavée ([Fig. 10](#)), tels que le Livarot, l'Epoisses et le Saint Nectaire. Parmi les espèces dominantes dans ces fromages figurent les levures *Geotrichum candidum* et *Debaryomyces hansenii*, et les espèces gram-négatives *H. citridevorans*, *Pse. nigrifaciens*, *Psy. cibarius/immobilis* et *Psy. translucens* [3, 4, 48, 232]. Ces fromages sont fabriqués à partir de la coagulation lactique ou mixte avec des ferments mésophiles, tels que *L. lactis* subsp. *cremoris* sans chauffage ou avec un chauffage léger (<40 °C) et sont principalement des fromages à pâte molle ([Fig. 1](#)). Par conséquent, la surface de ces caillés reste relativement humide, ce qui favorise probablement la croissance des bactéries halophiles. Dans le présent travail, nous avons eu pour approche de sélectionner des fromages fins et de déconstruire leurs croûtes microbiologiquement.

Il aurait sans doute été souhaitable de réaliser des mesures supplémentaires pour mieux caractériser les paramètres environnementaux qui ont permis le développement des différents microbiotes dans chaque fromage et pour comprendre les éventuelles successions d'espèces au cours de la production.

Notre travail a, cependant, fourni une « image » finale contenant, exhaustivement, les différents acteurs qui se sont développés à la surface des fromages. De plus, nous avons sauvegardé dans nos congélateurs un ensemble de souches isolées, qui pourraient être utilisées pour des expériences de reconstruction d'écosystèmes fromagers afin de mieux comprendre leur rôle dans ce produit. Par exemple, différentes souches d'espèces halophiles que nous avons isolées pourraient être testées en interaction entre elles et avec les levures citées précédemment dans des modèles de fromages afin d'effectuer un premier criblage des interactions positives (comme la croissance de la souche halophile stimulée par la présence de la levure) ou négatives (souche halophile inhibée).

En effet, il a été montré dans les fromages que les LAB pouvaient générer un large spectre de molécules telles que les acides organiques, le dioxyde de carbone, l'éthanol, le peroxyde d'hydrogène et le diacétyl, dont les actions antimicrobiennes sont bien connues [233]. Il en est sans doute de même pour les bactéries halophiles et il est intéressant de noter que dans notre étude ([Publication 5](#)), deux souches de *Psychrobacter* (FME6 et 1439) ont produit, en quantité importante, plusieurs acides organiques ainsi que du diacétyl, qui pourraient avoir une fonction d'interférence dans la croissance d'autres microorganismes présents dans les fromages. Ainsi, certaines molécules sécrétées par ces bactéries pourraient exercer des actions positives telles que la production de substrats ou de facteurs de croissance sur d'autres bactéries. Ces possibilités méritent d'être vérifiées, non seulement pour les bactéries contaminantes et pathogènes, mais aussi en présence d'autres espèces technologiques que l'on peut trouver à la surface des fromages, telles que *Brevibacterium aurantiacum*, *Brachybacterium tyrofermentans*, *Corynebacterium casei*, *Glutamicibacter arilaitensis* et *Staphylococcus equorum*. On peut également se demander si les échecs répétés dans l'isolement des souches *Pseudoalteromonas* sp. MAG-FME70 et *Marinomonas foliarum* MAG-FME69 pourraient être dus à une telle action d'une (ou plusieurs) bactérie(s) présente(s) dans l'écosystème fromager ?

Nous avons aussi remarqué que certaines espèces halophiles formaient des biofilms différents à la surface de nos modèles de caillé-agar, ce qui suggère la production d'autres type de composés

importants dans la construction des écosystèmes, comme les polysaccharides [234, 235]. Ces molécules pourraient avoir un rôle important non seulement pour la construction de l'écosystème, mais aussi pour la capacité de rétention d'humidité du fromage et sa texture. En outre, il a été démontré que de nombreuses bactéries halophiles environnementales synthétisent et accumulent souvent des polysaccharides extracellulaires (EPS) en tant que stratégies adaptatives face aux fortes pressions salines, qui diffèrent considérablement par leurs propriétés physiques, chimiques et structurelles [236].

Enfin, ces interactions, lors de leur développement, pourraient être explorées dans des modèles de fromage avec des analyses simultanées de métatranscriptomes (RNA-Seq) et de composés volatils (GC-MS), afin de mieux caractériser les facteurs impliqués dans ces interactions et le rôle des bactéries halophiles, d'un point de vue écologique mais aussi technologique. Il est important de noter que toutes les souches appartenant à la même espèce n'ont pas nécessairement le même comportement physiologique. Ce fait a, par exemple, été observé dans nos analyses des composés volatils ([Publication 5](#)), pour lesquels des souches appartenant à *Pseudoalteromonas nigrifaciens* présentaient des capacités de croissance, des valeurs de pH et de production de composés volatils, très distinctes entre elles.

b) Définir l'aptitude technologique des halophiles

Après avoir mieux compris le rôle et la place des halophiles dans l'écosystème des surfaces des fromages, il sera possible d'en définir leurs potentialités technologiques. Des tests préliminaires sur les propriétés fonctionnelles tel que celui que nous avons conduit ([Publication 5](#)), devraient aussi être pris en compte pour proposer différentes formulations qui pourraient être testées sur des modèles de fromages, dont les caractéristiques technologiques s'approcheraient de ceux mentionnées ci-dessus (fromages à pâtes molles et croûtes lavées). Faisant suite à l'élaboration de ces modèles de fromage, les analyses microbiologiques et métagénomiques, ainsi que les analyses de la composition du fromage (teneur en sel, humidité, matières grasses, protéines, pH) et les analyses biochimiques (activités enzymatiques, protéolyse, acides aminés libres) pourraient être effectuées à différents intervalles de temps d'affinage (entre 21 et 90 jours, par exemple). Afin de mieux cerner leurs propriétés et leurs potentialités technologiques, il serait également important de compléter ces études par des essais de détection de profils aromatiques pour associer « métabolisme » et « arôme ».

Les activités métaboliques produites par les souches halophiles peuvent être étudiées via des approches métabolomiques, à coupler avec des analyses métatranscriptomiques, comme mentionné précédemment, permettant l'exploration des gènes exprimés par ces bactéries, lors de l'affinage des fromages, et, en phase ultime, l'étude de leurs assignations fonctionnelles. Il serait également intéressant de découvrir si ces souches produisent des molécules inhibitrices et/ou ont un effet compétitif face aux bactéries pathogènes et bactéries d'altération. Par exemple, de nombreuses souches de bactéries lactiques (telles que *L. lactis* et *Lactiplantibacillus plantarum*), ajoutées comme ferments dans la production de fromages, produisent de la nisine et de la pédiocine [237-239], des bactériocines qui peuvent inhiber le développement de *Listeria monocytogenes* et de *Staphylococcus aureus*, qui comptent parmi les agents pathogènes les plus préoccupants pour l'industrie laitière [240]. Certaines souches de *Pseudoalteromonas* ont des activités antibactériennes et antivirales, comme *Pse. undina* [241], espèce qui a été détectée dans certains fromages, dans le cadre de cette étude ([Fig. 10](#)). De plus, une souche de *Pse. aliena* (LCCM 11207P), isolée à partir d'un produit fermenté coréen, a montré un effet inhibiteur significatif contre le *Vibrio harvery* [202].

D'un autre côté, certaines études considèrent que les bactéries halophiles sont impliquées dans l'altération des aliments et sont donc considérées comme indésirables dans les environnements de transformation des aliments [217-219]. C'est le cas, par exemple, d'espèces de *Pseudoalteromonas*, isolés à partir de la crevette brune, en particulier, *Pse. elyakovii* et *Pse. nigrifaciens*, qui ont été associées à la production de grandes quantités de composés soufrés (hydrure de soufre, méthane-thiol et disulfure de diméthyle), ainsi que d'acétone, d'acide acétique et d'ammoniac, ce qui peut entraîner un rejet sensoriel du produit [216]. De plus, cette même étude a indiqué que les espèces de *Pseudoalteromonas* et *Psychrobacter* ont la capacité de dégrader les lipides et d'hydrolyser les acides aminés et les protéines, ce qui peut augmenter le potentiel d'altération des aliments. Cependant, d'autres auteurs signalent que les composés volatils produits par les halophiles peuvent avoir un impact positif pendant l'affinage du fromage [215, 242, 243]. En outre, certaines souches de *Psychrobacter*, isolées à partir de la surface de fromages défectueux (espèces *Psy. pulmonis*, *Psy. aquimaris* et *Psy. okhotskensis*), ont été identifiées comme responsables des pigments violets sur les croûtes, ainsi que certaines espèces de *Proteus*. Ce défaut a été associé à la production de composés indigoïdes (indigo et indirubine), bien que la provenance de ses précurseurs métaboliques dans le fromage soit actuellement inconnue [244]. Dans ce cas,

les souches productrices de pigments ne sont pas mentionnées comme une menace pour les consommateurs, mais comme ayant un impact négatif sur la qualité du fromage.

c) S'assurer de l'innocuité des halophiles et de leur impact sur la santé

Bien que certaines espèces halophiles soient dominantes dans les croûtes de fromage et que la population en consomme régulièrement, ce qui suggère qu'elles ne sont pas pathogènes, il est important de s'assurer pleinement que la présence de ces microorganismes n'est pas nuisible à la santé humaine. La littérature scientifique est peu abondante sur ce sujet, mais apporte quand même quelques éléments concernant l'utilisation de quelques espèces d'halophiles des genres *Halomonas*, *Pseudoalteromonas* et *Psychrobacter* ayant des effets potentiels bénéfiques ou indésirables dans l'alimentation.

Concernant le genre *Halomonas*, Bourdichon *et al.* (2012) considèrent que l'espèce *H. elongata* joue un rôle technologique dans les viandes fermentées [17]. En effet, cette espèce peut être une bonne candidate comme ferment dans la saumure utilisée pour la préparation du jambon cru affiné (Brevet allemand, [DE 4035836 C2](#)), car certaines souches peuvent contribuer à améliorer la qualité sensorielle de ce produit [245]. En revanche, d'autres espèces d'*Halomonas* telles que *H. stevensii*, *H. hamiltonii* et *H. johnsoniae* ont été isolées dans un centre de dialyse sur deux patients souffrant de bactériémie, et pourraient donc constituer un facteur pathogène chez l'homme [246].

D'autre part, certaines espèces de *Pseudoalteromonas* produisent des toxines qui peuvent causer la mortalité ou l'empoisonnement des poissons (*Pe. piscicida* et *Pe. tetraodonis*) [247, 248]. En revanche, il serait intéressant de tester le rôle de certaines souches au niveau intestinal en faisant le parallèle avec la souche de *Pse. aliena* (LCCM 11207P), cité précédemment, qui a un effet inhibiteur sur le *Vibrio harvery*, un pathogène grave chez les poissons et les invertébrés, en mariculture [202].

Le genre *Psychrobacter* est rarement considéré comme opportuniste chez l'homme, mais une étude a indiqué que la souche 310 (proche de l'espèce *Psy. sanguinis*), isolée chez un patient atteint de méningite, possède plusieurs gènes de virulence, caractéristiques des bactéries pathogènes, notamment des gènes jouant un rôle dans la résistance aux antimicrobiens, l'invasion des cellules, l'absorption du fer et le système de sécrétion de type IV [249]. En outre, d'autres espèces telles que *Psy. phenylpyruvicus*, *Psy. pasteurii* et *Psy. piechaudii*, ont également été isolées chez l'homme et peuvent être responsables d'infections oculaires et d'endocardites [250, 251].

Enfin, il faut tenir compte, qu'au sein d'un même genre, chaque espèce peut avoir des caractéristiques très différentes, comme dans le cas du *Staphylococcus*. Par exemple, *S. aureus* est considéré comme un pathogène dangereux [252], tandis que *S. equorum* est un ferment avec une utilisation technologique dans l'affinage du fromage [253]. Heureusement, nous avons observé qu'aucune espèce isolée chez l'homme n'est étroitement associée aux génomes alimentaires ([Fig. 11](#), [Fig. S1-S5](#), [Annexe 3](#)), suggérant qu'il y a des différences significatives entre les souches présentes dans l'alimentation et celles détectées dans des environnements hospitaliers.

ANNEXE 1

1. Analyse génomique de deux souches halophiles marines

1.1. Contexte

L'intérêt que nous portons à l'origine potentielle des bactéries alimentaires halophiles au sein des milieux naturels nous a conduits à une collaboration avec Ifremer (Institut Français de Recherche pour l'Exploitation de la Mer). Dans le cadre de cette coopération, il a été fourni huit séquences d'ARNr 16S, issues d'espèces non définies de bactéries marines des genres *Halomonas*, *Psychrobacter* et *Pseudoalteromonas*. Ces séquences ont été placées dans un arbre phylogénétique avec les espèces fromagères isolées dans les [Publications 1](#) et [2](#), (voir [Figure 12](#) ci-dessous).

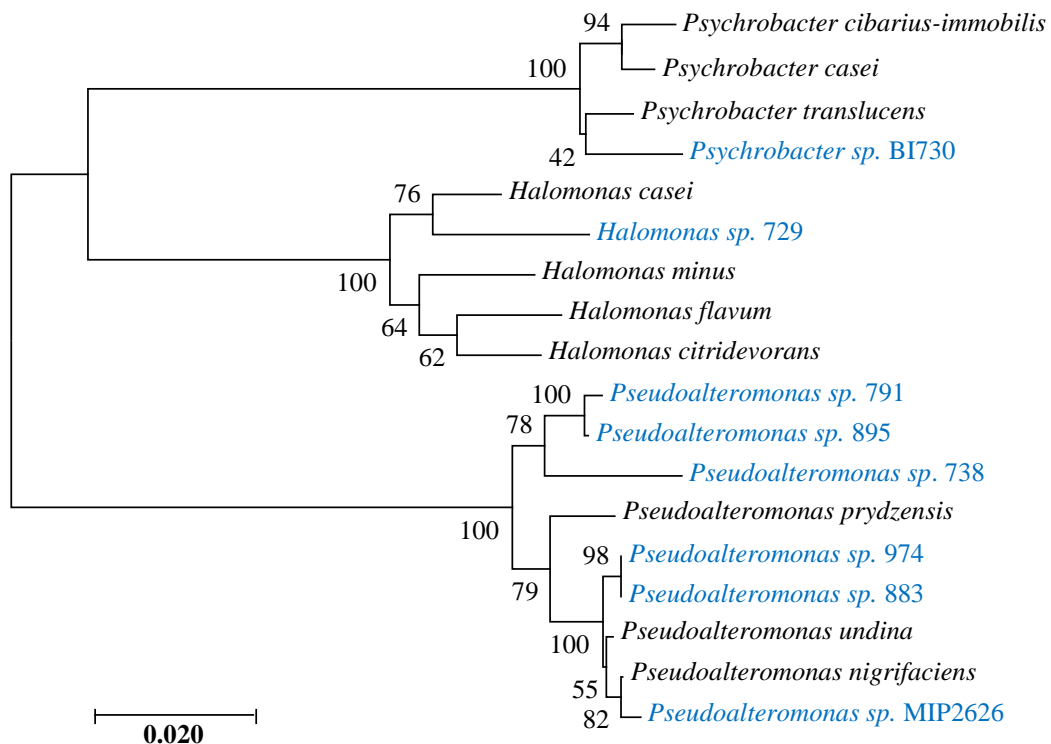


Figure 12. Arbre phylogénétique basé sur les alignements des gènes de l'ARNr 16S de souches marines (en bleu) et fromagères (en noir).

Parmi ces souches, *Pseudoalteromonas sp. MIP2626* et *Psychrobacter sp. BI730* ont été choisies pour effectuer une analyse génomique plus approfondie, fonction de leurs proximités avec des espèces fromagères.

Les résultats obtenus ont été publiés sous la forme d'un article de données, dans la revue *Data in Brief* et constituent la section 1.2 de l'Annexe 1.

1.2. Publication 6

Draft-genome sequence data and phylogenomic comparison of two marine-sourced bacterial strains *Pseudoalteromonas* sp. MIP2626 and *Psychrobacter* sp. BI730

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Abstract

Halophilic and psychrophilic marine bacteria are source of interesting bioactive molecules for biotechnology. We report here the whole-genome sequences of two of them, *Pseudoalteromonas* sp. MIP2626 isolated from tropical peeled shrimps and *Psychrobacter* sp. BI730 isolated from deep-sea hydrothermal vent. Sequencing of both genomes was performed by Illumina HiSeq platform (2×150 pb). *De novo* assemblies using SPAdes v3.9 generated 136 contigs for *Pseudoalteromonas* MIP2626 and 42 contigs for *Psychrobacter* BI730, representing a genome size of 3.9 Mb and 3.2 Mb, respectively. Phylogenetic based on 16S rRNA gene sequence and phylogenomic analyses were reported to compare the new sequences with *Pseudoalteromonas* and *Psychrobacter* representative strains available in the public databases. The genome sequences have been deposited at GenBank under the accession numbers JAATTW000000000 for *Pseudoalteromonas* sp. MIP2626 and JAATTV000000000 for *Psychrobacter* sp. BI730.

Keywords: Aquatic, Genomic, Phylogenetic analysis, Halophilic

Specifications Table

Subject	Molecular biology
Specific subject area	Microbiology and Genomics
Type of data	Draft genome sequences in FASTA format Tables Figures Mtsx tree files (phylogenetic 16S) Newick tree and svg files (phylogenomic)
How data were acquired	We extracted the genomic DNA using phenol-chloroform protocol [1] and whole genome sequencing was performed through the Illumina HiSeq platform
Data format	Raw and analyzed data
Parameters for data collection	Genomic DNAs were extracted from pure cultures of MIP2626 and BI730 strains
Description of data collection	Whole-Genome sequencing, assembly, annotation, phylogenetic and phylogenomic comparisons. Genomes were assembled with <i>de novo</i> assembly using SPAdes version 3.9 [2] and annotated with Rapid Annotations Subsystems Technology (RAST) [3]. Phylogenetic analysis of 16S rRNA were performed using MEGA 7.0.26 [4]. Multiple Alignment of Conserved Genomic Sequence With Rearrangements (MAUVE 2.4.0) was used for aligned the whole-genome sequences [5]. For the annotation and management of phylogenomic trees, we used iTOL v5 (http://itol.embl.de).
Data source location	<i>Pseudoalteromonas sp.</i> MIP2626 was isolated in France from tropical peeled shrimps in 2009 and <i>Psychrobacter sp.</i> BI730 from deep-sea hydrothermal vent site in Lau Basin, in the Southwestern Pacific Ocean, in 1989.
Data accessibility	The genome of the MIP2626 strain has been deposited in NCBI database under the Bioproject PRJNA604092, the Biosample SAMN13951749 and the genome accession number JAATTW000000000. The genome of the BI730 strain has been deposited in NCBI database under the Bioproject PRJNA604092, the Biosample SAMN13951813 and the genome accession number JAATTV000000000 All data are available in a public repository: Repository name: Draft-genome sequence data of <i>Pseudoalteromonas sp.</i> MIP2626 and <i>Psychrobacter sp.</i> BI730 Direct URL to data: http://dx.doi.org/10.17632/kbdfk2d7vy.1

Value of the Data

- The data contribute to describe the genomic diversity of the *Pseudoalteromonas* and *Psychrobacter* species;
- Genome sequence data of halophilic strains from marine origin are useful for comparative genomic analysis and highlight the genetic adaptation of these species in food ecosystems;
- Genome sequences provide new knowledge on *Pseudoalteromonas* and *Psychrobacter* species of biotechnological importance.

1. Data Description

Pseudoalteromonas and *Psychrobacter* species are Gram-negative moderate halophilic bacteria that could be isolated in different natural ecosystems like soil, salt lake and marine environment [6], but also in salted food such as seafood, processed meat and cheese [7–9]. As they are psychrophilic bacteria, both genera are interesting for biotechnological applications [10–13]. In this paper, we present the whole-genome sequences of two strains collected by Ifremer (Nantes, France) from marine samples. The first strain, *Psychrobacter* sp. BI730 was isolated in 1989 from a deep-sea hydrothermal vent site in Lau Basin, in the Southwestern Pacific Ocean, explored during BIOLAU oceanographic cruise. The second one, *Pseudoalteromonas* sp. MIP2626, was isolated in 2009 from tropical peeled shrimps.

Based on the homology of 16S rRNA gene sequences with those of closely type strains (Additional File 1), *Psychrobacter* sp. BI730 shares high similarity with *Psychrobacter nivimaris* 88/2-7^T (99.93% for a 1461 bp sequence) (Fig. 1). In the same way, *Pseudoalteromonas* sp. MIP2626 strain appears closely related to *Pseudoalteromonas nigrifaciens* KMM 661^T (100% for a 1458 bp sequence) and *Pseudoalteromonas haloplanktis* ATCC 14393^T (99.18% for a 1467 bp sequence) (Fig. 2).

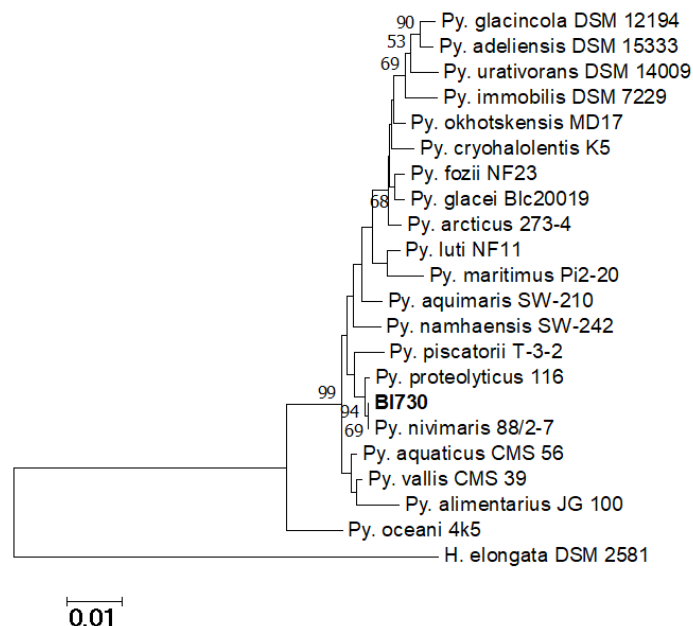


Figure 1. Phylogenetic tree of *Psychrobacter* genus, including *Psychrobacter* sp. BI730 and *Psychrobacter* type strains. The phylogenetic tree is based on 16S rRNA gene alignments obtained by MEGA 7.0.26 software using the neighbor-joining method. *Halomonas elongata* DSM 2581^T was used as the outgroup.

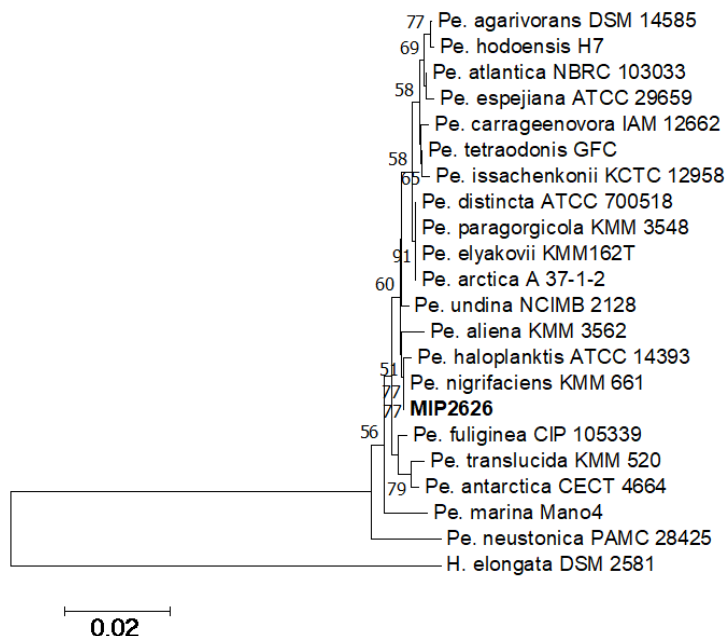


Figure 2. Phylogenetic tree of *Pseudoalteromonas* genus, including the *Pseudoalteromonas* sp. MIP2626 and *Pseudoalteromonas* type strains. The phylogenetic tree is based on 16S rRNA gene alignments obtained by MEGA 7.0.26 software using the neighbor-joining method. *Halomonas elongata* DSM 2581^T was used as the outgroup.

Whole-genomes of *Psychrobacter* sp. BI730 and *Pseudoalteromonas* sp. MIP2626 were sequenced through the Illumina HiSeq platform generating respectively 7,118,942 and 4,547,663 paired-end reads (2×150 bp) sequences. *De novo* assemblies yielded draft genomes with an average cover of 302.74 for *Psychrobacter* sp. BI730 and 168.00 for *Pseudoalteromonas* sp. MIP2626. Then, the contigs were filtered to keep those of length >300 bp and coverage >100 . *Psychrobacter* sp. BI730 showed 42 contigs, covering 3,263,843 bp, with 42.7% G+C content and N50 of 207,652 bp. It encodes 2,871 coding sequences classified in 291 subsystems and 46 RNAs. *Pseudoalteromonas* sp. MIP2626 showed 136 contigs, covering 3,988,911 bp, with 40.0% G+C content and N50 of 64,825 bp. It encodes 3,792 coding sequences classified by RAST in 354 subsystems and 97 RNAs.

Phylogenomic comparison based on type strains or reference genomes available in public databases was performed. Further information about the selected reference genomes are shown in Additional File 2. *Psychrobacter* sp. BI730 belongs to a group with several marine strains (Fig. 3). No ANI value over 95 % of BI730 strain with the reference genomes available was observed. *Pseudoalteromonas* sp. MIP2626 is closely related to both food and marine strains (Fig. 4). This

strain showed an ANI value higher than 95 % with the type strains *Pseudoalteromonas nigrifaciens* NCTC10691^T (97.84 %) and *Pseudoalteromonas translucida* KMM 520^T (96.1 %).

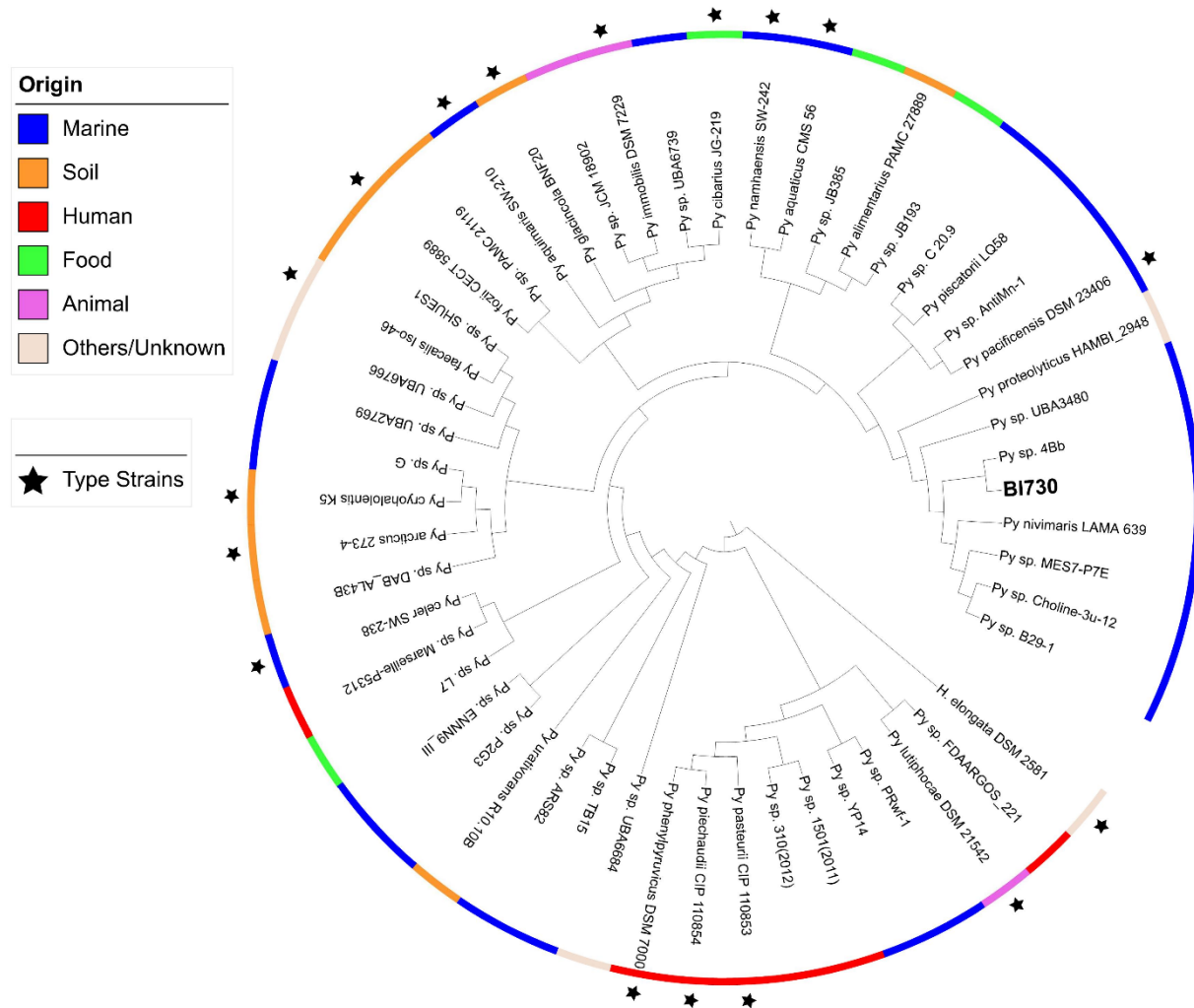


Figure 3. Phylogenomic comparison of *Psychrobacter* strains from diverse origins using the software Mauve 2.4.0 and visualized with iTOL v5. *Halomonas elongata* DSM 2581^T was used as the outgroup.

denaturation, 0.5 min at 56°C for primer annealing, 1.5 min at 72°C for elongation, and (iii) 5 min at 72°C to ensure final elongation. DNA amplicons were separated on 0.8% agarose gel, purified by using the ExoSAP-IT (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and sent for sequencing to the service provider (Eurofins Genomics, Ebersberg, Germany). Sequences were analyzed to obtain a preliminary taxonomic classification for each isolate.

We elaborated an initial phylogeny of the isolates BI730 and MIP2626 using the closest related 16S rRNA gene sequences generated by EzBiocloud blast (<https://www.ezbiocloud.net/>). The GenBank accession numbers of these species are shown in Additional File 1. Further phylogenetic analysis were performed using MEGA 7.0.26 software and trees were generated using the Neighbor-Joining algorithm with 1000 bootstrap iterations [4].

2.3. Genome sequencing and assembly

Whole genome sequencing was performed through the Illumina HiSeq platform at GATC-Biotech (Konstanz, Germany) The genomes were assembled with *de novo* assembly using SPAdes version 3.9 [2]. Then, the contigs were filtered to keep those of length >300bp and coverage >100. Annotations were produced using the Rapid Annotations using Subsystems Technology server [3].

2.4. Phylogenomic analysis

Representative complete genome sequences of *Psychrobacter* and *Pseudoalteromonas* were collected from the NCBI database. Information about these genomes such as isolation origin, Bioproject and BioSample numbers, and Average Nucleotide Identity (ANI) with BI730 and MIP2626 are shown in Additional File 2. ANI values, were determined by JSpeciesWS [14]. *Psychrobacter* and *Pseudoalteromonas* genome sequences selected from the public database were aligned with the BI730 and MIP2626 strains, respectively, using the software Progressive Mauve (version 2.4.0) with the default settings [5]. The phylogenomic trees were visualized with Interactive Tree of Life (iTOL v5) (<http://itol.embl.de>).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships which have, or could be perceived to have, influenced the work reported in this article.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:[10.1016/j.dib.2020.105898](https://doi.org/10.1016/j.dib.2020.105898).

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1.3. Conclusion

Concernant les données, nous avons décrit ici deux génomes marins appartenant aux genres *Pseudoalteromonas* et *Psychrobacter*. Malgré le séquençage génomique, il n'a pas été possible de les assigner à des espèces connues, selon les critères de l'ANI. Cela est la conséquence soit de l'absence de génomes d'espèces, définis dans la base de données qui sont proches de la souche *Psychrobacter sp.* BI730 (pas d'ANI>95% avec un génome de référence), soit du conflit existant avec la taxonomie de la souche *Pseudoalteromonas sp.* MIP2626 (c'est-à-dire deux espèces définies avec un ANI>95%). Cependant, la souche MIP2626 pourrait être définie comme appartenant à l'espèce *nigrifaciens*, car elle présente la valeur la plus élevée d'ANI (97.84%) avec la souche type *Pseudoalteromonas nigrifaciens* NCTC 10691^T. Il est intéressant de noter qu'il existe une grande diversité d'espèces *Psychrobacter* dans la base de données provenant de différentes origines (marine, humaine, alimentaire, animale, etc...), tandis que pour le genre *Pseudoalteromonas*, la majorité des espèces séquencées sont issues des environnements marins. De plus, les souches alimentaires identifiées pour ce dernier genre (souches JB197 et NCTC 10691^T) sont étroitement liées à la souche présentée dans cet article (MIP2626). Ce résultat suggère que l'origine des souches alimentaires peut être d'ordre environnemental. Ainsi, de manière générale, ces données contribuent à décrire la diversité génomique des espèces de *Pseudoalteromonas* et de *Psychrobacter* et peuvent faciliter l'étude de l'adaptation des espèces environnementales aux écosystèmes alimentaires.

ANNEXE 2

1. Méta-analyse de la communauté microbienne de fromages traditionnels brésiliens

1.1. Contexte

Les fromages artisanaux, produits au Brésil, sont largement appréciés et consommés. Cependant, la biodiversité des espèces naturellement présentes dans ces produits fermentés est encore loin d'avoir révélé tous ses secrets et son potentiel. Bien que de nombreuses études aient été consacrées à la caractérisation microbiologique de fromages brésiliens, à l'aide de méthodes de culture, classiques, ces techniques ne permettent pas d'avoir une vision complète du microbiote existant. Récemment, Kamimura *et al.* [254] ont réalisé une étude pionnière concernant la diversité bactérienne des fromages artisanaux de différentes régions du Brésil, en utilisant une approche métagénomique (séquençage du gène ARNr 16S). Ils ont identifié principalement les bactéries lactiques des genres *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc* et *Streptococcus*, ainsi que la présence de certains contaminants possibles des genres *Enterobacteriaceae* et *Staphylococcus*. Toutefois, cette étude s'est limitée à l'assignation des bactéries au niveau du genre et n'a pas exploré la communauté fongique existante dans les fromages brésiliens. Ainsi, compte tenu de l'importance économique, sociale et culturelle des fromages artisanaux produits au Brésil, la compréhension de leur microbiote complet devient nécessaire pour améliorer la qualité et la sécurité du produit. Pour cela, cette étude vise à :

- identifier le microbiote bactérien et fongique des croûtes et des cœurs de 23 fromages artisanaux, produits au Brésil, en utilisant des approches métagénomiques (amplification des gènes ARNr 16S et ITS) ;
- analyser les croûtes et cœurs de certains fromages avec la métagénomique *shotgun* ;
- identifier les espèces caractéristiques des fromages brésiliens.

Les résultats obtenus sont présentés sous la forme d'un article en cours de préparation, et devant constituer la partie 1.2 de l'Annexe 2.

1.2. Publication 7

Meta-analysis of five traditional Brazilian cheese varieties

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Abstract

In this study, we applied independent-culture methods to characterize the microbiota of 23 Brazilian artisanal cheeses, corresponding to five varieties (Araxá, Canastra, Serro, Colonial and Serrano). For assessing the bacterial and fungal diversity, we divided the rind and core of cheeses and, at first, we performed the high-throughput amplicon sequencing of 16S rRNA and ITS genes. The results showed a dominance of *Lactococcus lactis*, *Streptococcus thermophilus* and *Streptococcus infantarius* in the core of the cheeses and abundance of *Corynebacterium variabile* and of yeasts *Debaryomyces hansenii*, *Diutina catenulata*, *Kodamaea ohmeri*, *Trichosporon sp.* and *Moniella sp.* on the cheese rinds. Some rinds (n=9) and cores (n=6) were selected to improve the accuracy of species and diversity detection of Brazilian cheeses, using shotgun metagenomic sequencing. From the 15 metagenomes, we recovered 60 metagenomic-assembled genomes (MAGs), including 50 prokaryotes and 10 eukaryotes. With these data, we identified four potential new species belonging to *Brevibacterium*, *Corynebacterium* and *Streptococcus*, as well as three other strains belonging to the *Micrococcaceae* and *Lactobacillaceae* families, which could not be assigned at genus level. Additionally, we reconstituted yeast genomes of *Debaryomyces hansenii* and *Geotrichum candidum* from Brazilian cheese rinds. Nevertheless, six other fungal MAGs, representatives of two different species belonging to the *Saccharomycetales* class, did not have close references in the public database. The availability of these data opens the opportunity to identify the typical genomic traits of Brazilian strains, in addition to pointing out new potential species, which can influence the characteristics of cheeses produced in Brazil.

Keywords: metagenomics, microbial diversity, Brazilian fermented food, artisanal cheese, metagenome-assembled genomes, Brazil.

1 Introduction

Microbial communities are considered among the main determinants of the diversity and quality of cheese [1]. These ecosystems are formed by a wide variety of microorganisms (such as yeasts, moulds and bacteria), which can be added by humans, depending on the technology or desired characteristics, or can be inserted incidentally throughout the manufacturing process. Over the last few years, advances in molecular biology and the use of next-generation sequencing technology have made possible an accurate image of the biodiversity in food and their evolution during the production chain [2]. Currently, there are more than 1,400 different types of cheese in the world, with a wide variety of textures, flavours and aromas. These characteristics are attributed to the development of complex and specific microbial communities, depending on the technology and ingredients applied, as well as on local factors such as the origin of the milk and agricultural practices.

In Brazil, there are more than 30 varieties of artisanal cheeses that are produced in micro regions of the Southeast (Araxá, Campo das Vertentes, Cerrado, Canastra and Serro), South (Colonial and Serrano), Center (Caipira), North (Marajó) and Northeast (Butter and Curd) of the country [3]. The cultural patrimony and the *savoir-faire* of each type of traditional cheese, as well as its sensory characteristics, can be associated with its microbial richness. However, these cheeses are usually produced with raw milk, which can be a transmission route for pathogens such as *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* spp. and *Staphylococcus aureus* [4].

Although many studies have focused on the microbiological characterisation of these Brazilian fermented products using dependent-culture methods [5-9], these techniques fail to provide a complete perspective of the existent microbiota, because several species are poorly cultivate or require appropriate culture media. Recently, Kamimura *et al.* (2019) [10] conducted a pioneering study on the application of culture-independent methods in Brazilian cheeses. They demonstrated, by sequencing the 16S rRNA gene, the bacterial diversity of artisanal cheese cores from different regions of Brazil, and identified mainly the lactic acid bacteria of the genera *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Streptococcus*, as well as some possible contaminants such as *Enterobacteriaceae* and *Staphylococcus*. However, this study was limited to the bacterial assignation at the genus level and did not explore the fungal diversity.

Considering the economic, social and cultural importance of the artisanal cheeses produced in Brazil, the comprehension of their complete microbiota becomes important to improve the quality

and safety of them. Therefore, the focus of this study was to identify the bacterial and fungal diversity of rinds and cores of five different types of cheeses (Araxá, Canastra, Serro, Colonial and Serrano) by metagenetic methods (amplification of 16S rRNA and ITS genes) and by using the shotgun metagenomic sequencing. The scientific data generated here can be used to assist in the market expansion, ensuring the protection of historical and sanitary aspects, in addition to supporting the elaboration of technical regulations, legislation or standardization parameters for the different types of traditional Brazilian cheeses.

2 Methodology

2.1 Sample collection and DNA extraction

A total of 23 artisanal cheese were obtained from Southeast and South of Brazil from five varieties: Araxá, Canastra, Serro, Colonial and Serrano (Table 1). Samples were collected from local producers, artisan markets and fairs, and sent by post to France. The rinds were separated from the cores, using sterile knives, and both fractions were analyzed to obtain a more detailed overview of the microbial diversity for those cheeses. The samples were diluted 1:1 (w/v) in guanidinium thiocyanate 4M solution (Sigma-Aldrich, MO, USA) with Tris-HCl 0.1M and mixed in an Ultra Turrax T25 (Labortechnik) at 8,000 rpm for 2 min. The mixture was added of 10% N-Lauryl sarcosine (Sigma-Aldrich, MO, USA) in a proportion of 1:0.08 (v/v), vortexed and centrifuged at 4 °C, 14,000 g during 30 min. The fat and supernatant were eliminated and the pellet was used for DNA extraction using the protocol described by Almeida *et al.* (2014) [11]. DNA quality was visualized on 0.8% agarose gel and the quantification was measured with Qubit 2.0 fluorometer (Life Technologies) using Qubit dsDNA HS (High Sensitivity) Assay Kit.

2.2 PCR-amplification of 16S rRNA and ITS genes

Bacterial diversity was analyzed by sequencing the amplified region V3-V4 of the 16S rRNA gene using primers V3F (5'-ACGGRAGCWGCAGT-3') and V4R (5'-TACCAGGGTATCTAATCCT-3'). Additionally, fungal diversity was evaluated in rinds using ITS3 (5'-GCATCGATGAAGAACGCAGC-3') and ITS4 (5'-TCCTCCGCTTWTGWTWTGC-3') primers. The PCR was performed with MTP Taq DNA Polymerase (Sigma-Aldrich, St. Louis, MO, USA) and the cycling conditions were: 94°C for 1 min, followed by 30 cycles of amplification at 94°C for 1 min, 65°C for 1 min, and 72°C for 1 min, with a final extension step of 10 min at

72°C. The sequencing was performed with V3 Illumina MiSeq kit, as described in Poirier *et al.* (2018) [12], and fastq files were generated at the end of the run (MiSeq Reporter software, Illumina, USA).

Table 1. Metadata describing the 23 cheese samples.

Sample	Cheese	Region Brazil	Milk	Ripening*	Collected
C-01	Canastra	Southeast	Raw	Short	Artisan market
C-02	Canastra	Southeast	Raw	Short	Artisan market
C-03	Canastra	Southeast	Raw	Short	Artisan market
C-04	Canastra	Southeast	Raw	Short	Artisan market
C-05	Canastra	Southeast	Raw	Short	Artisan market
C-06	Araxá	Southeast	Raw	Long with mould in surface**	Artisan market
C-07	Araxá	Southeast	Raw	Short	Artisan market
C-08	Araxá	Southeast	Raw	Short	Producer
C-09	Araxá	Southeast	Raw	Short	Artisan market
C-10	Araxá	Southeast	Raw	Short	Artisan market
C-11	Serro	Southeast	Raw	Medium with mould in surface	Artisan market
C-12	Serro	Southeast	Raw	Short	Artisan market
C-13	Serro	Southeast	Raw	Medium with mould in surface	Artisan market
C-14	Serro	Southeast	Raw	Medium with mould in surface	Producer
C-15	Colonial	South	Raw	Short	Artisan market
C-16	Colonial	South	Raw	Short	Producer
C-17	Colonial	South	Raw	Short	Artisan market
C-18	Colonial	South	Raw	Short	Artisan market
C-19	Colonial	South	Pasteurized	Short	Public market
C-20	Serrano	South	Raw	Short	Producer
C-21	Serrano	South	Raw	Short	Fairs
C-22	Serrano	South	Raw	Short	Fairs
C-23	Serrano	South	Raw	Short	Public market

*Short: 15-30 days; medium: 30-60 days; long: >60 days

**Add *Geotrichum candidum* in surface

2.3 Metagenetic data analyses

The quality of the raw data was evaluated with FastQC [13] and the sequences were imported into the FROGS (Find Rapidly OTUs with Galaxy Solution) pipeline [14] to obtain the Operational Taxonomic Units (OTUs). The sequences were filtered by length (150–500 bp) and then pooled into OTUs with SWARM [15] with the distance parameter of 3. Chimeras were removed with VSEARCH [16] and we retained OTUs with at least 0.01% in the whole dataset. The OTUs were affiliated with SILVA 132 SSU databases [17] for bacteria and UNITE 8.2 for fungi. The alpha and beta diversity analyses were performed in R Studio v. 3.6.1 using the phyloseq package (v1.30.0) [18].

2.4 Taxonomic composition by shotgun metagenomics

From the metagenetic results, 15 cheese samples (nine surfaces and six core) were selected for a more in-depth analysis. The DNA of those cheeses were sequenced using Illumina HiSeq2500 technology at GATC-Biotech (Konstanz, Germany), which yielded between six and eight million paired-end reads of 150-nucleotide length. From the fastq file generates, we first estimated microbial composition by mapping the samples reads against the representative clade-specific marker catalog contained on the MetaPhlan tool v. 3.0.4 [19]. As an additional method for taxonomic profiling, we used a marker gene prediction, which allows binning of assembled metagenomic contigs without the need for reference sequences. Therefore, the reads were merged and de novo assembly was performed using SPAdes v. 3.9 [20]. Then, genes were predicted using Prodigal v. 2.6.3 and single copy marker genes are extracted using fetchMG v. 1.0 [21,22]. By default, fetchMG searched for 40 universal marker genes. We focused our research on the COG0012 (ychF gene) and blasts were performed on the all-available sequences in the NCBI databases on nucleic sequences and on the coding DNA sequences. Summary species composition plots were created in R v. 3.6.1 using the package ggplot2 v. 3.3.2. Additionally, all metagenomic reads were also mapped against *Bos taurus* genome with Bowtie2 [23] and visualized with Samtools flagstat [24].

2.5 Metagenome-assembled genome (MAG) quality and their taxonomic identification

Genome binning was performed using MetaBAT2-2.12.1 [25], with minimum contig size of 1,500 nucleotides and other default settings. The quality of the resulting prokaryotic bins were assessed with CheckM [26] and MAGs <80% completeness and/or >10% contamination were excluded. For species assignation of MAGs, we determined the Average Nucleotide Identity (ANI) with the closest reference using mummer-3.23 [27]. Moreover, for eukaryotes, we predicted the genes of MAGs and species closely related using Prodigal v. 2.6.3 and then we searched for 40 universal marker genes using fetchMG v. 1.0 [21,22]. From this analysis, ten marker genes were identified in all MAGs (ychF, argS, rpsL, rpsB, rplK, rplC,rplB, rplF, rplP and leuS). These proteins were concatenate to construct a phylogeny. The phylogenetic analyses were performed using ClustalX 2.1 [28] and MEGA7 [29] and using the Neighbor-Joining method [30] with 1,000 bootstrap replicates [31].

2.5 Data availability

Raw sequences of amplification of 16S rRNA and ITS genes and raw metagenomic reads were deposited to European Nucleotide Archive (ENA) under the BioProject ID PRJNA693797. The MAGs are available in a public repository under the URL: <http://dx.doi.org/10.17632/nj3dbvg6ty.1>

3 Results and Discussion

3.1 Taxonomic diversity of Brazilian cheeses using amplicon sequencing approaches

The bacterial diversity of 23 Brazilian core and rind cheeses was assessed with 16S rRNA amplicon sequencing and the sequences were clustered in 157 bacterial OTUs, which taxonomic assignment was possible up to the species level in the majority of the cases (Fig. 1). In these analyses, we discard the sample C-02 due to its low depth of sequences. The mainly species present in core of cheeses were the lactic acid bacteria (LAB) commonly used as starters, such as *Lactococcus lactis* and *Streptococcus thermophilus*. We observed also a high abundance of *Streptococcus infantarius* in two samples of Araxá cheeses, from Brazilian Southeast. Previous studies showed that this species is highly prevalent and predominant among LABs in spontaneously fermented African dairy products from cow, goat and camel milk [32,33]. *S. infantarius* was also isolated from European and South American cheeses [34,35], including the Coalho cheese from Brazil [36,37]. Additionally, in the core of C-R22 sample, we observed the predominance of the species *Kluyvera cryocrescens* (pattern of an *E. coli*-like strain, [38]), indicating the poor quality of this cheese.

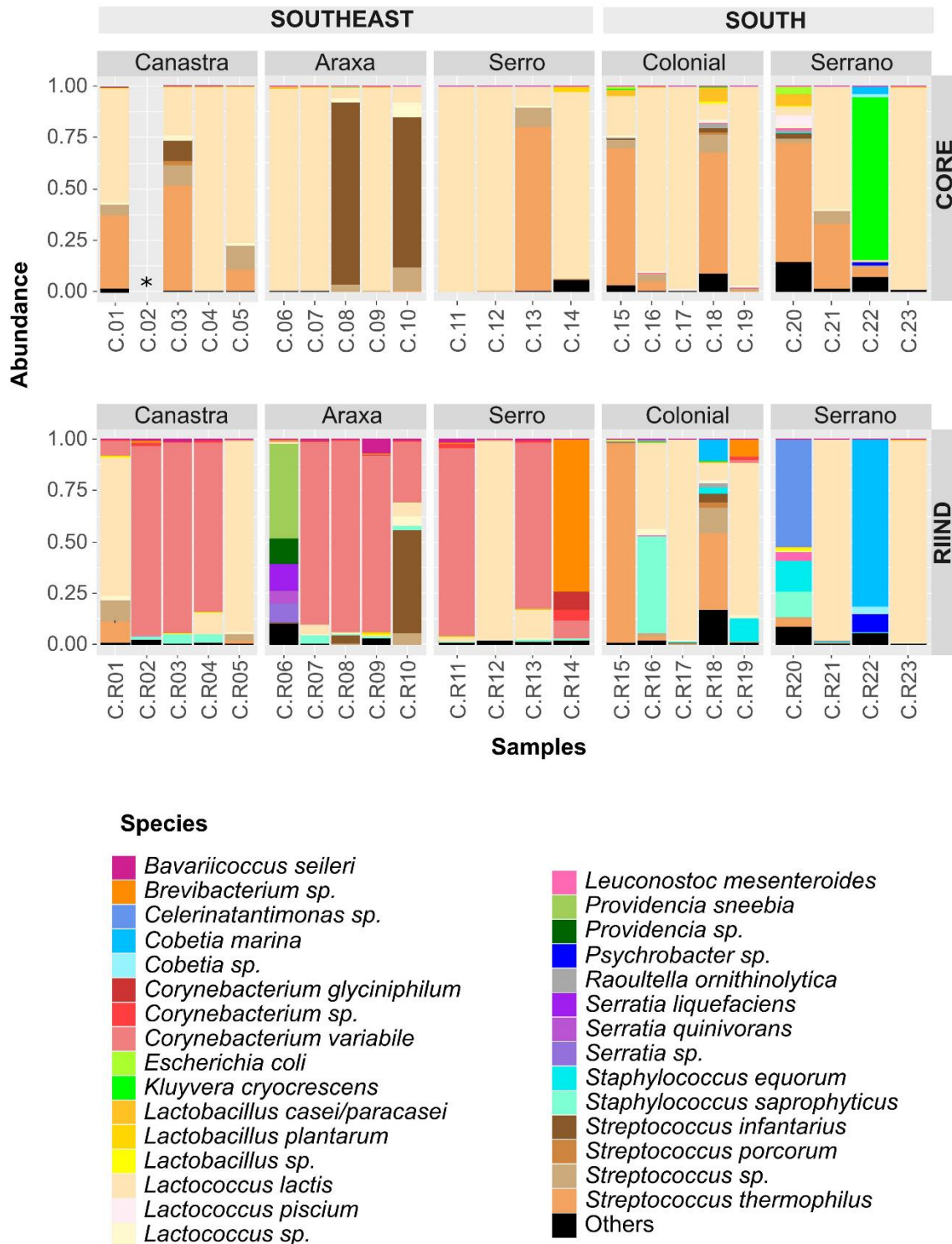


Figure 1. Bacterial plot depicting the relative abundance of the 30 main species found in 23 Brazilian cheese core and rinds.

The asterisk (*) indicates low depth of the sequences.

Concerning the bacteria present on rinds, some Brazilian cheese surfaces are predominantly of core species (Fig. 1). Indeed, most of these cheeses have a short ripening time (15-30 days, Table 1), which may be insufficient, coupled with the conditions of the aging cave, for the growth of surface bacteria. Overall, the *Corynebacterium variabile* is the predominant species in Southeast cheese rinds. This species is well known for being part of the complex microflora on the surface of ripened cheeses [39,40], which possibly was added as a starter. Some samples of rinds presented particular composition, as the C-R06 sample (Araxá cheese), which was dominated by *Providencia* spp. and *Serratia* spp. Both genera have already been isolated from cheeses [41,42]. Additionally, the C-R14 sample (Serro cheese) presented dominance of *Brevibacterium* and strains of this genus are often used as surface-ripening cultures in different cheese types [43], but they can also be adventitious bacteria. On the cheese surfaces from South, the composition of bacteria is highly varied among producers (Fig. 1), indicating lack of standardization. In two Serrano surface samples, we observed high abundance of *Celerinatantimonas* sp. (C-R20) and *Cobetia marina* (C-R22), two bacteria that belong to the *Gammaproteobacteria* class and probably play a spoilage role in cheese. Finally, alpha-diversity analysis showed greater richness of bacterial species ($p < 0.05$) in the South samples when compared to the Southeast of Brazil, and PCoA showed the distances of the cheeses from the two regions with a common bacterial communities (Fig. S1).

Parallel to the bacterial analysis, the fungal diversity of 17 rind samples (samples that amplifications were successful) was evaluated with the ITS amplicon sequencing and the sequences were grouped into 34 OTUs of fungi. The most abundant and/or frequent fungi species identified in cheese from Southeast were *Diutina catenulata*, *Kodamae ohmeri*, *Trichosporon* sp. and *Moniliella* sp. (Fig. 2). These genera/species have already been mentioned in dairy products [44,39,41]. Interestingly, two samples from Southeast presented particular fungi microbiota, corresponding to the same rinds with different bacterial compositions (Fig. 1). It included the CR-06, that was dominated by *Geotrichum candidum* and probably its presence facilitate the dispersal of *Serratia* and *Providencia* species and shape the diversity of this cheese rind [45]. Additionally, the C-R14 sample showed a dominance of *Acremonium citrinum* and *Microascus* (*Scopulariopsis*) *brevicaulis*, genera already mentioned in the Wolfe *et al.* (2014) study [44], who evaluated 137 cheese rinds. Curiously, these fungi are usually present in cheese rinds with *Brevibacterium* dominance, as in this study. *Debaryomyces hansenii* was the predominantly yeast on the cheese rinds from South (Colonial and Serrano) and this species is commonly dominant on cheese rind

varieties around the world [39,46]. One sample from Colonial cheese (C-R16) was dominated by *Penicillium commune*, one of the most common fungi spoilage moulds on cheese rinds [47]. Finally, the alpha and beta-diversity analyses demonstrated that species richness and distances between fungal communities were particularly close between the South and Southeast regions (Figure S2).

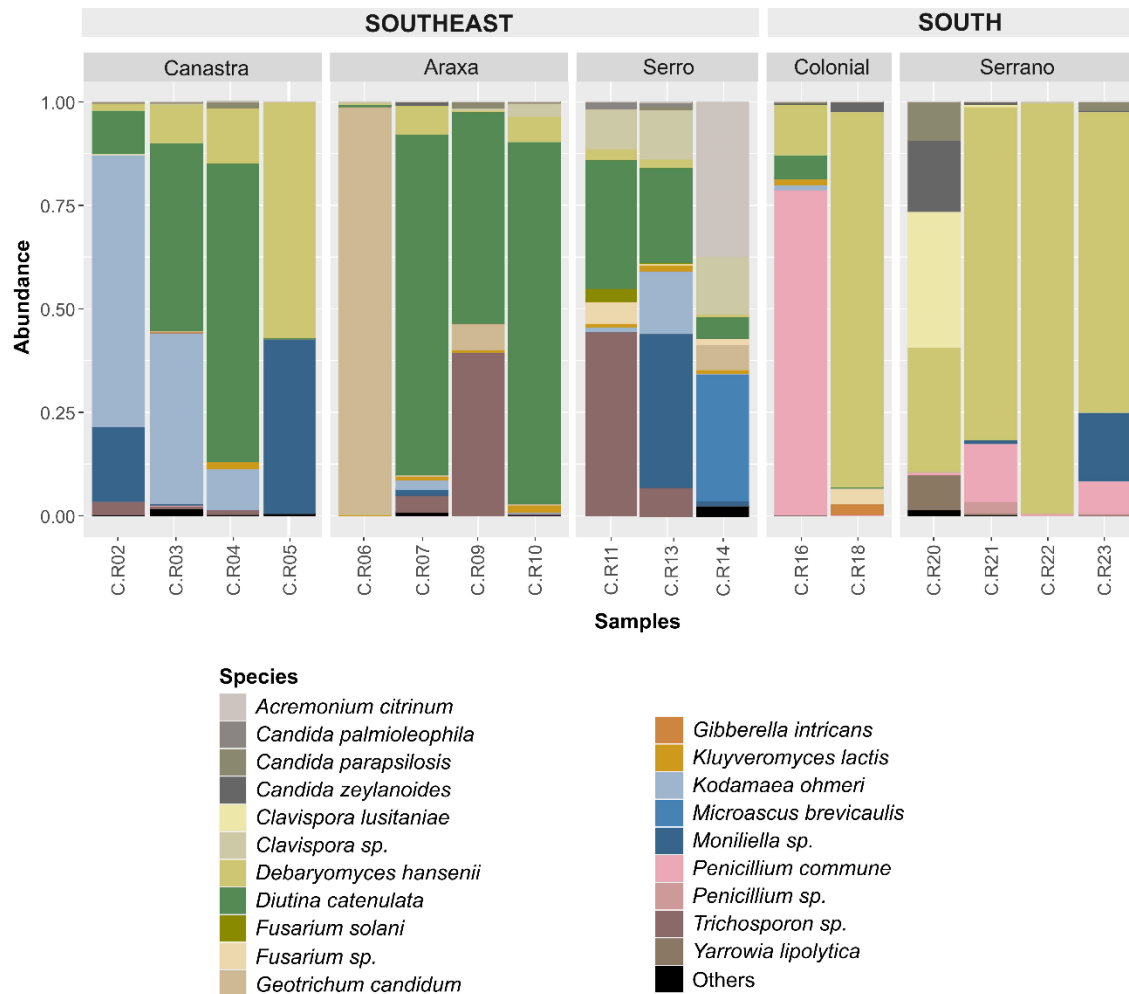


Figure 2. Fungal plot depicting the relative abundance of the 20 main species found in 17 Brazilian cheese rinds.

3.3 Microbial composition using shotgun-metagenomic approach

From the results of the sequencing of 16S rRNA and ITS genes, we selected the most distinct composition of six core and nine cheese rinds and then we performed a shotgun metagenomics sequencing of their DNAs. This analysis allowed a more reliable taxonomy for the species present

in the samples, as well as the proportion of bacteria and fungi together. Initially, the taxonomical composition of the 15 cheese samples was determined using MetaPhlAn. From this analysis, a total of 128 bacterial species and only one fungal species (*Candida parapsilosis*) were observed in the cheeses evaluated (Table S1). Overall, the bacterial results of our metagenomes were in accordance with the 16S rRNA amplicon methodology. However, ITS amplicon approach showed larger fungal diversity, and we assumed that this tool could have a lack of eukaryotic reference genomes. Then, we applied a marker gene analysis in our metagenomic dataset. As results, 56 bacterial species and seven fungal species were identified in the 15 cheeses (Table S2) and their microbial composition is shown in Fig. 3. We observed a more accurate taxonomy with this method, with the dominance of *Streptococcus thermophilus*, *S. infantarius* subsp. *infantarius* and *Lactococcus lactis* subsp. *lactis* in cheese cores and *Corynebacterium variabile* and *Staphylococcus saprophyticus* on rinds. We also pointed out a low relative abundance of eukaryotes in relation to bacteria on cheese rinds, and the predominant species of fungi in Brazilian cheese could not be assigned.

Concerning consumer risks, several samples, especially for the core (C-13, C-15 and C-18), presented more than 30% of reads mapped on the *Bos taurus* genome (Table S3). For this reason, these samples have low sequencing depth and consequently the taxonomic composition is less accurate (Fig. 3, marked with an asterisk). The high presence of animal reads in some metagenomes could be associated with the presence of cow somatic cells in the milk, which indicates herd health problems [48]. Furthermore, we identified the presence of *S. aureus* in the C-R02 sample (abundance relative of 0.86%, Table S2), indicating a potential risk of enterotoxin production and foodborne outbreaks.

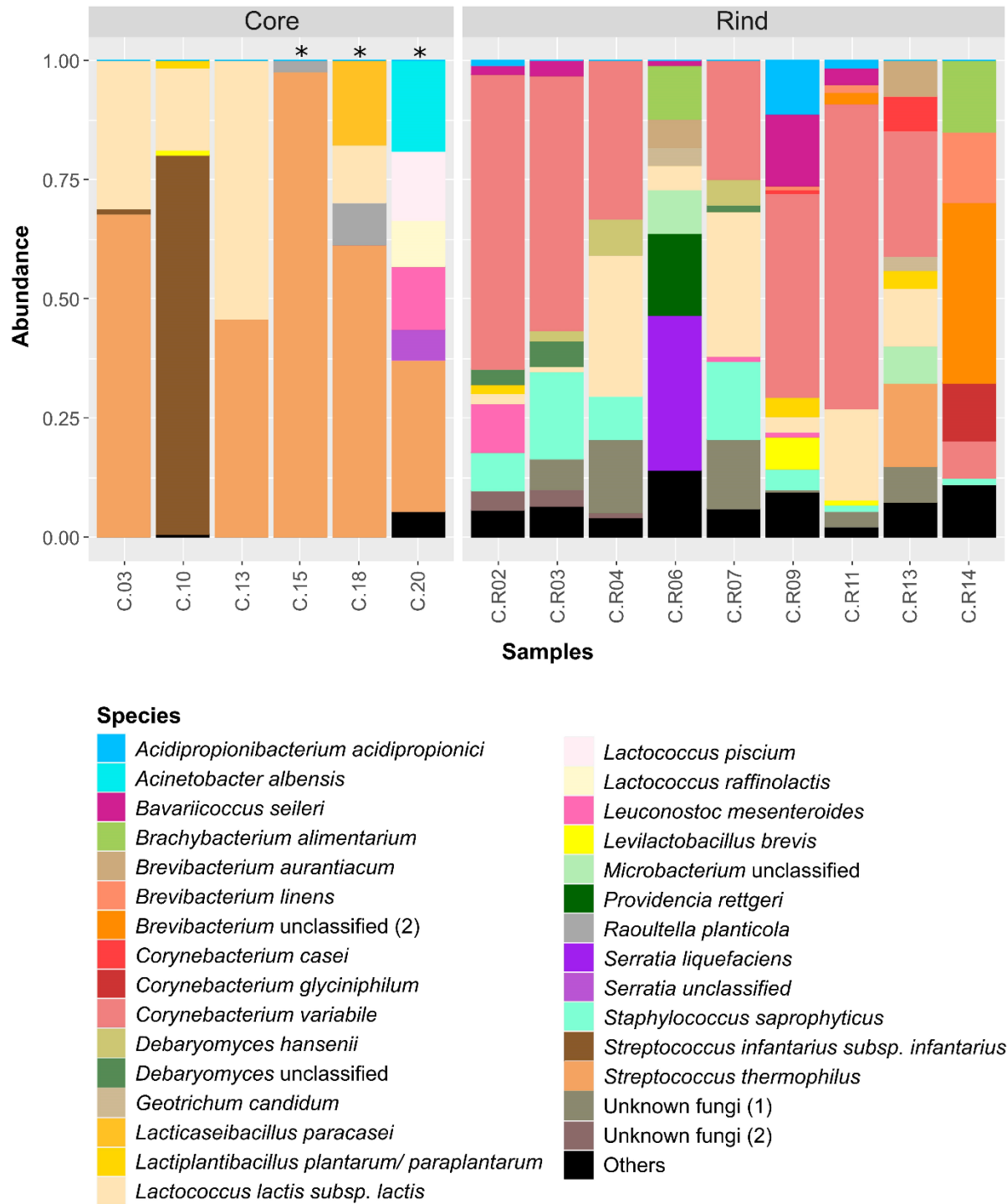


Fig. 3. The relative abundance of microbial composition of 15 Brazilian cheeses (six core and nine rinds). Results were based on ychF marker gene, extracted from the assembled metagenomes. The asterisks (*) indicates low depth of the sequences.

3.4 MAGs from Brazilian traditional cheeses

From the 15 metagenomes sequenced, a total of 50 prokaryotic metagenome-assembled genomes (MAGs) with high-quality (over 80% complete and less than 10% contamination) were reconstituted (Table S4). The majority of MAGs correspond to *Lactococcus lactis* subsp. *lactis* (10) and *Corynebacterium variabile* (8). Moreover, six MAGs represent four potential novel species, mainly from the C-R14 sample, since they have less than 95% average nucleotide identity (ANI) with reference genomes, it includes two species of *Brevibacterium*, one *Streptococcus* and one *Corynebacterium*. Three others prokaryotic MAGs, belonging to *Micrococcaceae* and *Lactobacillaceae* families could not be identified at the genus level (ANI<80%), suggesting they are characteristic bacteria of Brazilian cheeses. Additionally, ten fungal species were reconstituted (Table S5), being two *Debaryomyces hansenii*, two *Geotrichum candidum* and six other MAGs without closely related reference strains. Those last ones are representatives of two different species (Fig. S3). Therefore, the lack of reference genomes closely related to MAGs obtained in this study shows that the biodiversity of Brazilian cheeses still has many secrets, which can contribute to the organoleptic, cultural and historical characteristics of these artisanal fermented products.

4 Conclusion

The data obtained in this study expand and qualify the metagenomic database of Brazilian cheeses. Furthermore, these data can contribute to improve the production practices (quality and safety) and the acquisition of this know-how can support agro-industries and local economies. Additionally, the identification of potential new species opens the opportunity to isolate them and improve understanding at genomic and technological levels, since they can play an important role in the ecology and sensory aspects of Brazilian cheeses. At the same time as new strains are characterized, possibilities arise to develop inoculants maintaining cultural and historical identities, as well as to study microbial interactions and assist to establish standards of quality for cheeses produced in Brazil.

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Supplementary Material

The supplementary Tables S1-S5 can be found at <http://dx.doi.org/10.17632/7vc3vndc3r.1>.

Table S1. The relative abundance of microbial composition of 15 cheese metagenomes from Brazil using MetaPhlAn taxonomic assignment method.

Species	C-03	C-10	C-13	C-15	C-18	C-20	C-R02	C-R03	C-R04	C-R06	C-R07	C-R09	C-R11	C-R13	C-R14
Actinomycetaceae_bacterium_sk1b4	0	0	0	0	0	0	0.353	0.269	0	0	0	2.951	0.451	0.033	0
Bifidobacterium_mongoliense	0	0	0	0	0.28	0	0	0	0	0	0	0	0	0	0
Corynebacterium_casei	0	0	0	0	0	0	0.311	0	0.296	0.059	0.148	0.512	0.199	12.35	0.009
Corynebacterium_flavescens	0	0	0	0	0	0	0.05	0	0.037	0	0.129	0.013	0	0	0
Corynebacterium_glyciniphilum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.228
Corynebacterium_nuruki	0	0	0	0	0	0	0	0	0	0	0	0	0.019	0	2.987
Corynebacterium_sp_CNJ_954	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5.642
Corynebacterium_variabile	0	0.006	0.024	0	0	0	72.48	72.26	53.58	0.365	37.94	53.62	80.19	44.21	25.6
Dietzia_timorensis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.14
Brevibacterium_aurantiacum	0	0	0	0	0	0	0	0	0	2.983	0	0	0	4.435	0.033
Brevibacterium_iodinum	0	0	0	0	0	0	0	0.008	0.007	0	0	0.01	0.006	0	0
Brevibacterium_linens	0	0	0	0	0	0	0	0	0	7.101	0	0	0	8.913	0.418
Brachybacterium_alimentarium	0	0	0	0	0	0	0	0	0	17.77	0	0.005	0.041	0	47.22
Kocuria_kristinae	0	0	0	0	0.201	0	0.346	0.621	0	0	0.059	0	0	0	0
Rothia_sp	0	0	0	0	0.107	0.435	0	0	0	0	0	0	0	0	0
Acidipropionibacterium_acidipropionici	0	0	0	0	0	0	0.878	0.407	0	0	0	14.01	1.553	0.806	0
Propionibacterium_freudenreichii	0	0	0	0	0	0	0	0	0	1.328	0	0.34	0	1.137	0
Nocardiopsis_alba	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.131
Chryseobacterium_bovis	0	0	0	0	0.047	0.482	0	0	0	0	0	0	0	0	0
Chryseobacterium_haifense	0	0	0	0	0	0.149	0	0	0	0	0	0	0	0	0
Chryseobacterium_sp_FP211_J200	0	0	0	0	0	0.638	0	0	0	0	0	0	0	0	0
Myroides_odoratimimus	0	0	0	0	0	0	0	0	0	0.015	0	0	0	0	0
Kurthia_sp_11kri321	0	0	0	0.124	0	0.137	0	0	0	0	0	0	0	0	0
Macrococcus_caseolyticus	0	0	0	0	0	0.039	0.098	0	0	0	0	0	0	0	0
Staphylococcus_argenteus	0	0	0	0	0	0	0.008	0	0	0	0	0	0	0	0
Staphylococcus_aureus	0	0	0.003	0	0	0	1.084	0	0	0	0	0	0	0	0
Staphylococcus_carnosus	0	0	0	0	0	0	0.045	0	0	0	0	0	0	0	0
Staphylococcus_equorum	0	0	0	0	0	0	0	0	0	0	0	0.03	0	0	0
Staphylococcus_lentus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.963
Staphylococcus_nepalensis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.84
Staphylococcus_saprophyticus	0	0	0	0	0	0	8.594	19.65	12.39	0	23.6	4.415	0.545	0	1.587
Staphylococcus_xylosus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4.212
Carnobacterium_divergens	0	0	0	0	0	0.401	0	0	0	0	0	0	0	0	0
Carnobacterium_maltaramaticum	0	0	0	0	0	1.939	0	0	0	0	0	0	0	0	0
Marinilactibacillus_psychrotolerans	0	0	0	0	0	0	0	0	0	0.074	0	0	0	0	0
Bavariicoccus_seileri	0	0	0	0	0	0	0.717	1.907	0	0.271	0.448	5.08	0.989	0	0
Enterococcus_devriesei	0	0	0	0	0	0	0	0	0	0.121	0	0	0.01	0.024	0
Enterococcus_dispar	0	0	0	0	0	0	0	0	0	0	0	0	0.001	0	0
Enterococcus_faecalis	0	0	0	0.083	0.038	0	0	0	0	1.592	0	0.04	0	0.485	1.868
Enterococcus_italicus	0	0	0	0	0.181	0.241	0.007	0	0	2.793	0	0	0	0	0
Enterococcus_malodoratus	0	0	0	0	0	0	0	0	0	0.287	0	0	0	0.134	0
Tetragenococcus_halophilus	0	0	0	0	0	0	0	0	0	0	0	0.474	0	0.094	0
Lactobacillus_acidipiscis	0	0	0	0	0	0	0	0	0	0	0	0.031	0	0	0
Lactobacillus_brevis	0.155	0.435	0.213	0	0	0	0.454	0.687	0.484	0.304	0.098	5.628	0.707	0.284	0
Lactobacillus_curvatus	0	0	0	0	0	0	0.453	1.66	0.017	0.171	0.408	1.939	0.055	0.028	0
Lactobacillus_delbrueckii	0	0	0	0.568	0.426	0	0	0	0	0	0	0	0	0	0
Lactobacillus_futsaii	0	0	0	0	0	0	0.011	0	0	0	0	0.02	0	0	0
Lactobacillus_kefiranofaciens	0	0.235	0	0	0	0	0	0	0	0	0	0	0	0	0
Lactobacillus_kefiri	0	0	0	0	0	0	0	0	0.074	0	0	0	0	0	0
Lactobacillus_otakiensis	0	0.051	0	0	0	0	0	0	0	0	0	0	0	0	0
Lactobacillus_parabuchneri	0	0.044	0.036	0	0.838	0	0	0	0	0	0	0	0	0.111	0
Lactobacillus_parafarraginis	0	0	0	0	0	0	0	0	0.016	0	0	0	0	0	0
Lactiplantibacillus_paraplantarum	0.183	0.553	0	0	0	0	0.65	0.707	0	0.088	0	6.536	0.674	0	0
Lactobacillus_paucivorans	0	0	0	0	0	0	0	0	0	0	0	0.168	0	0	0
_Lactiplantibacillus plantarum	0.033	1.494	1.017	0	0	0	0.488	0	0.441	0	1.439	0.045	0.157	6.032	0.12

Lactobacillus_rhamnosus	0	0.006	0	0	0.079	0	0	0	0	0	0	0.434	0	0	
Lactobacillus_rossiae	0	0.004	0	0	0	0	0	0	0	0	0	0	0	0	
Lactobacillus_senmaizukei	0	0	0	0	0	0	0	0	0	0	0	0.189	0	0	
Lactobacillus_tuccei	0	0	0	0	0	0	0	0	0	0	0	0.062	0	0	
Lactobacillus_versmoldensis	0	0	0	0	0	0	0.064	0	0	0	0	0.03	0.015	0	
Pediococcus_parvulus	0	0	0	0	0	0	0	0.004	0	0	0	0.009	0	0	
Pediococcus_pentosaceus	0.072	0	0	0	0	0	0	0	0	0	0	0	0	0	
Pediococcus_sp	0	0	0	0	0	0	0	0.02	0	0	0	0.012	0	0	
Leuconostoc_citreum	0	0	0	0	0	0	0.019	0	0	0	0	0	0	0	
Leuconostoc_garlicum	0	0	0	0	0	0.123	0	0	0	0	0	0	0	0	
Leuconostoc_gelidum	0	0	0	0	0	1.107	0	0	0	0	0	0	0	0	
Leuconostoc_lactis	0	0	0	0	0	0.25	0	0	0	0	0	0	0	0	
Leuconostoc_mesenteroides	0	0	0	0.257	2.522	3.744	11.29	0	0	0.051	0.991	0.939	0	0.063	
Leuconostoc_pseudomesenteroides	0	0	0.412	0	0	0	0	0.033	0	0	0	0	0.292	0	
Weissella_jogaejeotgali	0	0	0	0	0	0	0	0.12	0.076	0	0.756	0.038	0	0	
Weissella_paramesenteroides	0	0	0	0	0	0	0	0.048	0	0	0	0	0	0	
Weissella_thailandensis	0	0	0	0	0	0	0	0.141	0.029	0	0.659	0.004	0	0	
Lactococcus_chungangensis	0	0	0	0	0	0.138	0	0	0	0	0	0	0	0	
Lactococcus_garvieae	0	0	0	0	0.978	0.666	0	0	0	0	0	0	0	0	
Lactococcus_lactis	61.98	19.5	80.7	4.051	7.951	6.091	1.553	1.237	31.4	5.335	33.02	2.738	13.52	10.76	2.937
Lactococcus_petauri	0	0	9E-04	0	0.453	0.304	0	0	0	0	0	0	0.009	0	
Lactococcus_piscium	0	0	0	0	4.415	13.75	0	0	0	0	0	0	0	0	
Lactococcus_raffinolactis	0	0	0	0	1.398	8.108	0	0	0	0	0	0	0	0	
Streptococcus_agalactiae	0	0.247	0.026	0	0	0	0	0	0	0	0	0	0	0	
Streptococcus_hongkongensis	0	0	0	0	0.03	0.047	0	0	0	0	0	0	0	0	
Streptococcus_infantarius	0.955	77.38	0	0	0.372	0	0	0.089	0	0	0	0	0	0	
Streptococcus_lutetiensis	0	0.046	0	0	0	0	0	0	0	0	0	0	0	0	
Streptococcus_macedonicus	0	0	0	0.239	0	1.219	0	0	0	0	0	0	0	0	
Streptococcus_parauberis	0	0	0	0	0.937	6.394	0	0	0	0	0	0	0	0	
Streptococcus_plurimalium	0	0	0	0	0.154	0	0	0	0	0	0	0	0	0	
Streptococcus_salivarius	17.71	0	5.317	0	2.478	0	0	0.043	0	0	0	0	0	1.736	
Streptococcus_thermophilus	18.91	0	12.19	93.61	56.6	37.43	0	0.028	0	0	0	0	0	2.334	
Streptococcus_uberis	0	0	0	0	0.019	0	0	0	0	0	0	0	0	0	
Veillonella_parvula	0	0	0	0	0.847	0	0	0	0	0	0	0	0	0	
Enhydrobacter_aerosaccus	0	0	0	0	1.324	0.384	0	0	0	0	0	0	0	0	
Aeromonas_allosaccharophila	0	0	0	0	0.806	0	0	0	0	0	0	0	0	0	
Aeromonas_caviae	0	0	0	0	0.74	0	0	0	0	0	0	0	0	0	
Aeromonas_media	0	0	0	0	1.934	0	0	0	0	0	0	0	0	0	
Citrobacter_freundii	0	0	0	0	0.3	0	0	0	0	0	0	0	0	0	
Citrobacter_youngae	0	0	0	0	0.976	0.636	0	0	0	0	0	0	0	0.016	
Enterobacter_cloacae_complex	0.004	0	0.011	0.265	0.104	0	0	0	0	0	0	0	0	0	
Escherichia_coli	0	0	0	0.317	0	5.997	0	0	0	0	0.009	0	0	0	
Klebsiella_michiganensis	0	0	0	0	0.215	0.522	0	0	0	0	0	0	0	0	
Klebsiella_oxytoca	0	0	0	0	0.093	0	0	0	0	0	0	0	0	0	
Klebsiella_pneumoniae	0	0	0	0	0.349	0	0	0	0	0	0	0	0	0	
Klebsiella_variicola	0	0	0	0.052	0	0	0	0	0	0	0	0	0	0	
Kluyvera_cryocrescens	0	0	0	0.393	0	0	0	0	0	0	0	0	0	0	
Raoultella_ornithinolytica	0	0	0	0.046	8.201	1.705	0	0	0	0	0	0	0	0.16	
Raoultella_terrigena	0	0	0	0	0	0	0	0	0	0.096	0	0	0	0	
Hafnia_alvei	0	0	0	0	1.291	0	0	0	0	0	0	0	0	0	
Hafnia_paralvei	0	0	0	0	0	0	0	0	0	1.956	0	0	0	0	
Morganella_morganii	0	0	0	0	0	0	0	0	0	0	0	0	0	0.072	
Proteus_hauseri	0	0	0	0	0	0	0	0	0	0.571	0	0	0	0.384	
Proteus_mirabilis	0	0	0	0	0	0	0	0	0	0	0	0	0	3.48	
Proteus_vulgaris	0	0	0	0	0	0	0	0	0	4.327	0	0	0	1.493	
Providencia_rettgeri	0	0	0	0	0	0	0	0	0	0	0	0	0	0.155	
Serratia_liquefaciens	0	0	0	0	0	0	0	0	0	49.91	0	0	0	0	
Serratia_marcescens	0	0	0	0	0.048	0.668	0	0	0	0.45	0	0	0	0.025	
Serratia_rubidaea	0	0	0	0	0	0	0.008	0	0	0.257	0	0	0	0.016	
Halomonas_campaniensis	0	0	0	0	0	0	0	0	0	0	0	0.05	0	0	
Marinomonas_sp_MWYL1	0	0	0	0	0	0	0	0	0	0.007	0	0	0	0	
Acinetobacter_berezinae	0	0	0	0	0	0.543	0	0	0	0	0	0	0	0	
Acinetobacter_johnsonii	0	0	0	0	0.218	2.265	0	0	0	0	0	0	0	0	
Acinetobacter_junii	0	0	0	0	0	0.514	0	0	0	0	0	0	0	0	
Acinetobacter_parvus	0	0	0	0	0.023	0	0	0	0	0	0	0	0	0	
Acinetobacter_ursingii	0	0	0.046	0	0	1.309	0	0	0	0	0	0	0	0	
Moraxella_osloensis	0	0	0	0	1.578	0.907	0	0	0	0	0	0	0	0	
Pseudomonas_fluorescens_group	0	0	0	0	0.261	0.725	0	0	0	0	0	0	0	0	
Pseudomonas_putida_group	0	0	0	0	0	0	0	0	0	0.38	0	0	0	0	
Vibrio_alginolyticus	0	0	0	0	0	0	0	0	0	0	0	0	0.264	0	
Vibrio_furnissii	0	0	0	0	0	0	0	0	0	0	0	0	0.118	0	
Stenotrophomonas_maltophilia	0	0	0	0	0.192	0	0	0	0	1.317	0	0	0	0	
Stenotrophomonas_rhizophila	0	0	0	0	0	0	0	0	0	0.019	0	0	0	0	
Candida_parapsilosis	0	0	0	0	0	0	0.033	0.055	1.147	0	0.309	0.105	0	0	

Table S2. The relative abundance of microbial composition of 15 cheese metagenomes from Brazil using ychF marker gene assignment.

Species	C-03	C-10	C-13	C-15	C-18	C-20	C-R02	C-R03	C-R04	C-R06	C-R07	C-R09	C-R11	C-R13	C-R14
<i>Acidipropionibacterium acidipropionici</i>	0	0	0	0	0	0	0.929	0	0	0	0	11.08	1.54	0	0
<i>Acidipropionibacterium unclassified</i>	0	0	0	0	0	0	0	0	0	0	0	0	1.543	0	0
<i>Acinetobacter albensis</i>	0	0	0	0	0	19	0	0	0	0	0	0	0	0	0
<i>Ancrocorticia populi</i>	0	0	0	0	0	0	0.861	0	0	0	0	2.659	0	0	0
<i>Arthrobacter unclassified</i>	0	0	0	0	0	0	3.23	0	0	0	0	0	0	0	0
<i>Bavariicoccus seileri</i>	0	0	0	0	0	0	1.859	3.113	0	0.871	0	15.23	3.575	0	0
<i>Brachybacterium alimentarium</i>	0	0	0	0	0	0	0	0	0	11.43	0	0	0	0	14.9
<i>Brachybacterium paraconglomeratum</i>	0	0	0	0	0	0	0	0	0	0	0	0.881	0	0	0
<i>Brevibacterium aurantiacum</i>	0	0	0	0	0	0	0	0	0	5.952	0	0	0	7.381	0
<i>Brevibacterium linens</i>	0	0	0	0	0	0	0	0	0	0	0	0.792	1.537	0	14.95
<i>Brevibacterium unclassified (1)</i>	0	0	0	0	0	0	0	1.002	0	0	0	0	0	0	0
<i>Brevibacterium unclassified (2)</i>	0	0	0	0	0	0	0	0	0	0	0	0	2.443	0	37.9
<i>Corynebacterium casei</i>	0	0	0	0	0	0	0	0	0	0	0	0.78	0	7.297	0
<i>Corynebacterium glyciniphilum</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12.12
<i>Corynebacterium variabile</i>	0	0	0	0	0	0	61.97	53.47	33.15	0	24.81	42.72	64.06	26.48	7.707
<i>Debaryomyces hansenii</i>	0	0	0	0	0	0	0	2.374	7.696	0	5.379	0	0	0	0
<i>Debaryomyces unclassified</i>	0	0	0	0	0	0	3.335	5.223	0	0	1.478	0	0	0	0
<i>Dietzia timorensis</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1.829
<i>Enterococcus faecalis</i>	0	0	0	0	0	0	0	0	0	1.005	0	0	0	0	0
<i>Enterococcus italicus</i>	0	0	0	0	0	0	0	0	0	2.381	0	0	0	0	0
<i>Geotrichum candidum</i>	0	0	0	0	0	0	0	0	0	3.658	0	0	0	2.86	0
<i>Hafnia alvei</i>	0	0	0	0	0	0	0	0	0	1.309	0	0	0	0	0
<i>Kluyveromyces lactis</i>	0	0	0	0	0	0	0	0	2.774	0	1.947	0	0	1.783	0
<i>Kocuria unclassified</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1.63
<i>Lactocaseibacillus paracasei</i>	0	0	0	0	17.75	0	0	0	0	0	0	0	0	0	0
<i>Lactiplantibacillus plantarum/ paraplantarum</i>	0	1.62	0	0	0	0	1.713	0	0	0	0	4.156	0	3.712	0
<i>Lactiplantibacillus unclassified</i>	0	0	0	0	0	0	0	0	0.905	0	0	0	0	0	0
<i>Lactobacillus curvatus</i>	0	0	0	0	0	0	0	2.076	0	0	0.894	1.834	0	0	0
<i>Lactobacillus huananensis</i>	0	0	0	0	0	0	0	0	0	0	0	2.638	0	0	0
<i>Lactobacillus kefiranofaciens</i>	0	0.387	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Lactobacillus rhamnosus</i>	0	0	0	0	0	0	0	0	0	0	0	0	0.621	0	0
<i>Lactococcus lactis subsp. lactis</i>	31.17	17.15	54.33	0	11.95	0	2.119	1.028	29.58	5.137	30.39	3.166	19.13	12.18	0
<i>Lactococcus piscium</i>	0	0	0	0	0	14.49	0	0	0	0	0	0	0	0	0
<i>Lactococcus raffinolactis</i>	0	0	0	0	0	9.857	0	0	0	0	0	0	0	0	0
<i>Lactococcus unclassified</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1.65
<i>Lentilactobacillus hilgardii</i>	0	0.293	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Leuconostoc mesenteroides</i>	0	0	0	0	0	13.01	10.21	0	0	0	1.131	1.005	0	0	0
<i>Levilactobacillus brevis</i>	0	1.142	0	0	0	0	0	0	0	0	0	6.853	1.078	0	0
<i>Microbacterium unclassified</i>	0	0	0	0	0	0	0	0	0	9.112	0	0	0	7.701	0
<i>Moraxellaceae bacterium</i>	0	0	0	0	0	5.486	0	0	0	0	0	0	0	0	0
<i>Propionibacterium freudenreichii</i>	0	0	0	0	0	0	0	0	0	0	0	0.723	0	2.612	0
<i>Proteus mirabilis</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	2.991	0
<i>Proteus vulgaris</i>	0	0	0	0	0	0	0	0	0	2.851	0	0	0	0	0
<i>Providencia rettgeri</i>	0	0	0	0	0	0	0	0	0	17.18	0	0	0	0	0
<i>Pseudomonas putida</i>	0	0	0	0	0	0	0	0	0	1.335	0	0	0	0	0
<i>Raoultella planticola</i>	0	0	0	2.294	8.88	0	0	0	0	0	0	0	0	0	0
<i>Rothia kristinae</i>	0	0	0	0	0	0	0.682	1.154	0	0	0	0	0	0	0
<i>Serratia liquefaciens</i>	0	0	0	0	0	0	0	0	0	32.54	0	0	0	0	0
<i>Serratia unclassified</i>	0	0	0	0	0	6.629	0	0	0	0	0	0	0	0	0
<i>Sphingomonas unclassified</i>	0	0	0	0	0	0	0	0	0.414	0	0	0	0	0	0

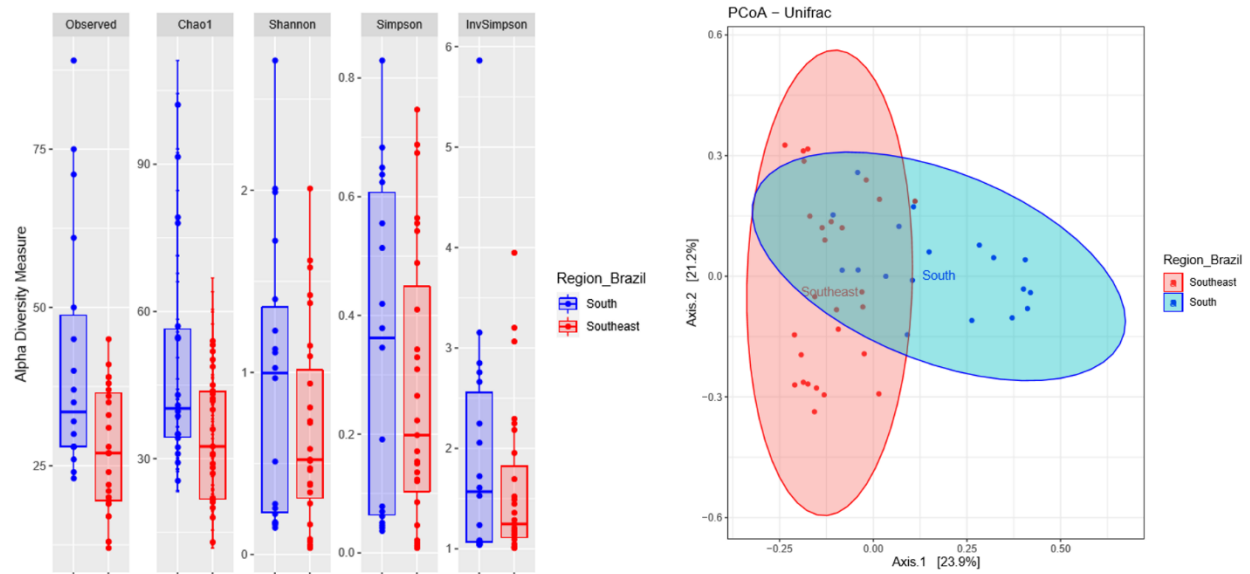
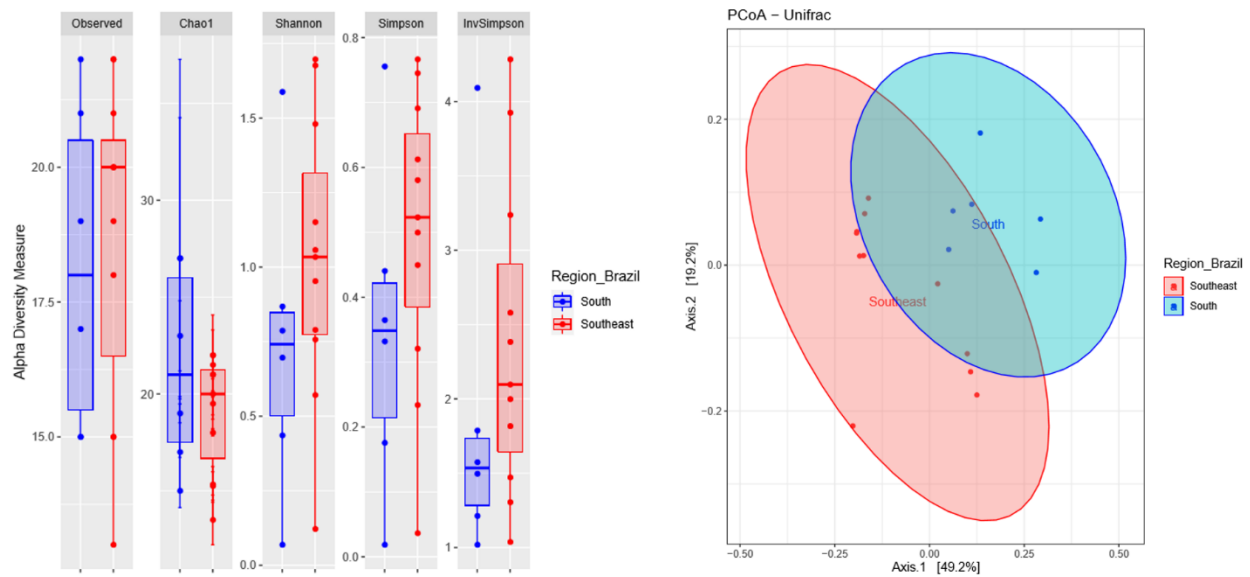
Staphylococcus aureus	0	0	0	0	0	0	0.863	0	0	0	0	0	0	0	0
Staphylococcus lentus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1.633
Staphylococcus nepalensis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.979
Staphylococcus saprophyticus	0	0	0	0	0	0	8.295	18.33	9.061	0	16.44	4.17	1.356	0	1.384
Staphylococcus xylosus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.312
Stenotrophomonas maltophilia	0	0	0	0	0	0	0	0	0	5.232	0	0	0	0	0
Streptococcus infantarius subsp. infantarius	0.899	79.4	0	0	0	0	0	0	0	0	0	0	0	0	0
Streptococcus thermophilus	67.93	0	45.67	97.71	61.42	31.53	0	0	0	0	0	0	0	17.71	0
Tetragenococcus unclassified	0	0	0	0	0	0	0	0	0	0	0	0.717	0	0	0
Unknown fungi (1)	0	0	0	0	0	0	0	6.453	15.32	0	14.38	0.601	3.114	7.29	0
Unknown fungi (2)	0	0	0	0	0	0	3.937	3.613	1.092	0	0	0	0	0	0
Unknown fungi (3)	0	0	0	0	0	0	0	1.131	0	0	0	0	0	0	0
Weissella jogaejeotgali	0	0	0	0	0	0	0	1.033	0	0	3.151	0	0	0	0

Table S3. Percentage (%) of *Bos Taurus* reads aligned in each cheese metagenome.

	Samples	% Reads aligned with <i>Bos taurus</i>
CORE	C-03	6.94
	C-10	1.16
	C-13	9.69
	C-15	31.65
	C-18	70.77
	C-20	76.42
RIND	C-R02	0.29
	C-R03	0.09
	C-R04	1.52
	C-R06	0.09
	C-R07	1.3
	C-R09	0.06
	C-R11	0.33
	C-R13	0.6
	C-R14	0.12

Table S4. Quality of prokaryotic MAGs and their ANIs with the closest reference genome found in the NCBI database.

MAG ID	Completeness	Contamination	Strain Heterogeneity	Reference strain	ANI (%)	Aligned nucleotides (%)
C-R09.bin.5	99.67	0	0	Acidipropionibacterium acidipropionici ATCC 4875	98.39	[89.52]
C-R03.bin.7	98.91	0	0	Bavariicoccus seileri WCC 4188	98.12	[85.30]
C-R11.bin.5	97.13	0.55	0	Bavariicoccus seileri WCC 4188	98.57	[91.37]
C-R09.bin.3	96.17	0.68	100	Bavariicoccus seileri WCC 4188	97.8	[74.46]
C-R02.bin.9	90.71	1.54	20	Bavariicoccus seileri WCC 4188	98.54	[88.92]
C-R06.bin.12	100	0.64	50	Brachybacterium alimentarium 908_11	98.99	[91.23]
C-R14.bin.9	95.55	3.28	66.67	Brachybacterium alimentarium 908_11	98.86	[90.70]
C-R13.bin.5	99.71	0	0	Brevibacterium aurantiacum SMQ-1420	97.81	[88.03]
C-R11.bin.7	94.86	7.11	40	Brevibacterium linens ATCC 9172	81.49	[60.10]
C-R14.bin.5	87.65	2.81	71.43	Brevibacterium linens ATCC 9172	83.78	[63.54]
C-R14.bin.10	93.05	3.25	80	Brevibacterium oceanii BBH7	81.15	[65.11]
C-R13.bin.8	93.47	0.88	0	Corynebacterium casei LMG S-19264	98.52	[88.49]
C-R14.bin.7	89.7	2.29	20	Corynebacterium glyciniphilum AJ-3170	88.03	[76.19]
C-R13.bin.7	98.87	0.45	0	Corynebacterium variabile DSM 44702	99.13	[93.60]
C-R07.bin.11	97.97	0.9	33.33	Corynebacterium variabile DSM 44702	99	[96.10]
C-R02.bin.10	97.82	1.95	16.67	Corynebacterium variabile DSM 44702	99	[93.29]
C-R11.bin.11	97.3	0.54	0	Corynebacterium variabile DSM 44702	99.11	[94.04]
C-R03.bin.6	97.07	0.5	50	Corynebacterium variabile DSM 44702	99.26	[96.34]
C-R09.bin.6	96.4	0.65	66.67	Corynebacterium variabile DSM 44702	99.3	[96.53]
C-R14.bin.2	96.38	1.28	50	Corynebacterium variabile DSM 44702	99.07	[94.76]
C-R04.bin.8	96.17	0.5	100	Corynebacterium variabile DSM 44702	99.36	[97.46]
C-R06.bin.9	92.5	3.2	5.56	Enterococcus italicus DSM 15952	98.7	[87.96]
C-R02.bin.1	92.75	1.03	0	Galactobacter caseinivorans JZ R-183	72.65	[36.24]
C-R13.bin.11	96.21	2.98	0	Lactiplantibacillus plantarum DSM 20174	99.04	[94.57]
C-10.bin.2	95.58	4.98	38.1	Lactiplantibacillus plantarum DSM 20174	98.5	[92.64]
C-R09.bin.11	94.16	1.09	0	Lactobacillus curvatus JCM 1096	98.86	[89.79]
C-R11.bin.2	100	0.5	100	Lactococcus lactis subsp. lactis II1403	99.28	[86.71]
C-10.bin.1	99.62	0	0	Lactococcus lactis subsp. lactis II1403	99.13	[87.68]
C-13.bin.2	99.62	0	0	Lactococcus lactis subsp. lactis II1403	99.3	[89.31]
C-R13.bin.13	99.62	5.41	4.55	Lactococcus lactis subsp. lactis II1403	98.76	[84.78]
C-03.bin.1	99.25	0.38	100	Lactococcus lactis subsp. lactis II1403	99.4	[90.12]
C-R09.bin.10	96.6	2.17	0	Lactococcus lactis subsp. lactis II1403	99.24	[88.85]
C-R04.bin.1	94.72	0	0	Lactococcus lactis subsp. lactis II1403	99.22	[85.98]
C-R07.bin.3	94.72	0	0	Lactococcus lactis subsp. lactis II1403	99.27	[86.61]
C-R02.bin.5	94.5	0.94	25	Lactococcus lactis subsp. lactis II1403	99.42	[91.64]
C-R06.bin.11	90.98	0	0	Lactococcus lactis subsp. lactis II1403	99.32	[90.78]
C-R02.bin.7	100	1.05	50	Leuconostoc mesenteroides ATCC 8293	98.85	[90.53]
C-R09.bin.9	82.74	0.99	33.33	Levilactobacillus brevis ATCC 367	73.52	[56.00]
C-R13.bin.1	93.13	1.11	16.67	Microbacterium excoecariae CBS5P-1	77.63	[44.68]
C-R13.bin.10	92.84	2.24	62.5	Proteus mirabilis HI4320	96.13	[85.63]
C-R06.bin.10	91.62	1.35	33.33	Proteus vulgaris ATCC 49132	99.63	[94.95]
C-R06.bin.5	97.21	7.21	17.65	Providencia rettgeri RB151	98.7	[85.62]
C-R06.bin.4	98.47	4.17	21.43	Serratia liquefaciens ATCC 27592	98.35	[91.11]
C-R07.bin.7	99.45	0	0	Staphylococcus saprophyticus ATCC 15305	99.36	[94.05]
C-R04.bin.7	98.9	0	0	Staphylococcus saprophyticus ATCC 15305	99.51	[94.66]
C-R09.bin.8	98.9	0	0	Staphylococcus saprophyticus ATCC 15305	99.62	[95.08]
C-R06.bin.7	94.43	4.92	32.26	Stenotrophomonas maltophilia SM 866	96.29	[86.80]
C-13.bin.1	89.12	0.5	100	Streptococcus salivarius ATCC 27945	93.77	[82.97]
C-R13.bin.3	90.51	0.75	0	Streptococcus salivarius ATCC 27945	93.78	[74.37]
C-R07.bin.8	98.19	2.34	12.5	Weissella jogaejeotgali FOL01	98.25	[86.40]

Table S5. Eukaryotic MAGs aligned with closely related reference genomes.**Figure S1.** Bacterial species richness in cheese samples in the South and Southeast Brazilian regions (A) and Principal Coordinates Analysis (PCoA) based on Unifrac distances among bacterial communities of the two regions (B).**Figure S2.** Fungal species richness in cheese samples (A) and Principal Coordinates Analysis (PCoA) based on Unifrac distances among bacterial communities of the two regions (B).

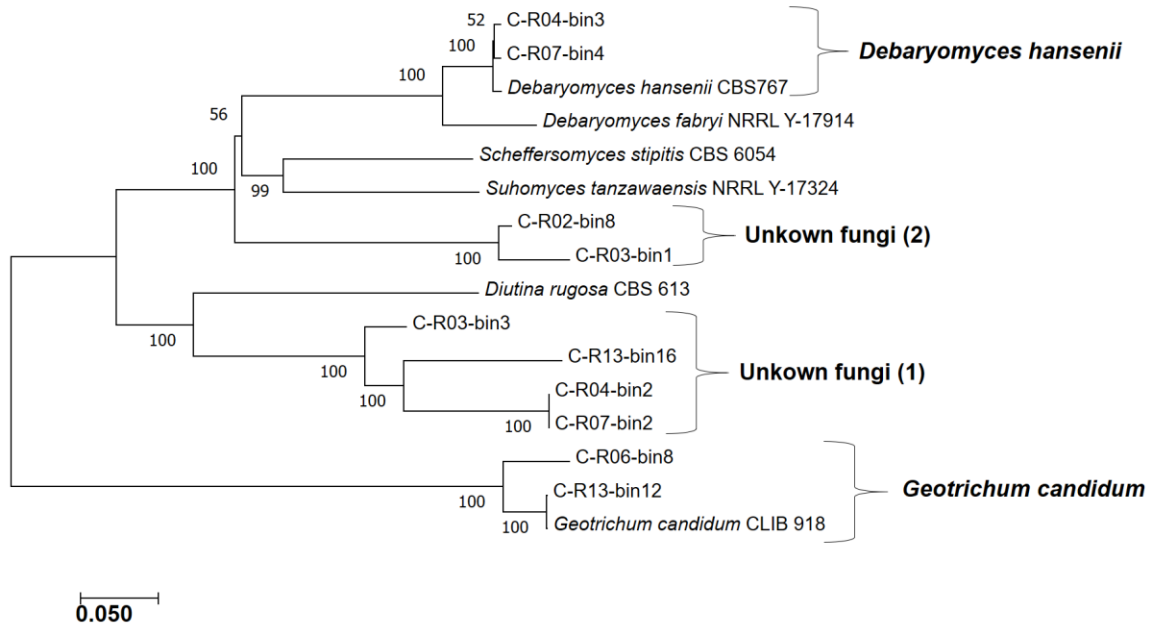


Figure S3. Phylogeny of eukaryotic MAGs and closely related references based on ten amino acid (ychF, argS, rpsL, rpsB, rplK, rplC, rplB, rplF, rplP and leuS). The tree was build with Neighbor-Joining method with 1,000 bootstrap replicates.

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1.3. Conclusion

Cette étude nous a permis d'évaluer, par des approches métagénomiques et métagénomiques, la *microbiota* de cinq variétés de fromages brésiliens, produites au Sud-est (Araxá, Canastra, Serro) et au Sud (Colonial et Serrano) de ce pays. Les principales espèces présentes dans les cœurs de ces fromages correspondaient à des bactéries lactiques communément utilisées comme ferments, telles que *Lactococcus lactis* subsp. *lactis* et *Streptococcus thermophilus*. Il est intéressant de noter que deux échantillons du fromage Araxá contenaient également une grande quantité de *Streptococcus infantarius*. D'autre part, trois échantillons de cœurs de fromages du Sud ont présenté plus de 30% de leurs *reads* métagénomiques, mappés sur le génome de la vache, ce qui pourrait suggérer des problèmes de santé chez ces animaux. Les différences entre microorganismes identifiés dans les fromages des deux régions sont plus apparentes au sein de leurs croûtes, où existait une prédominance de *Corynebacterium variabile* dans les fromages du Sud-est, alors que la distribution des bactéries était très variée dans les croûtes du Sud, ce qui indique un manque de standardisation. De plus, nous avons noté une prédominance de la levure *Debaryomyces hansenii* dans les fromages du Sud et, en particulier, deux espèces fongiques inconnues dans les croûtes des fromages produites au Sud-est du Brésil. A partir des analyses métagénomiques *shotgun*, nous avons obtenu la liste de 60 génomes assemblés par métagénomique (MAG), dont 50 procaryotes et 10 eucaryotes, qui peuvent être utilisés pour mieux comprendre les caractéristiques typiques des souches brésiliennes. De plus, la disponibilité de ces données ouvre la possibilité d'isoler spécifiquement ces nouvelles espèces pour améliorer nos connaissances aux niveaux génomique et technologique, car elles peuvent jouer un rôle important dans l'écologie et les aspects sensoriels des fromages brésiliens. Parallèlement à la caractérisation de nouvelles souches, ces données sont à même d'établir des normes de qualité pour les fromages produits au Brésil, en préservant leurs identités culturelles et historiques.

ANNEXE 3

Table S1. Méta-données de 114 métagénomés de croûte de fromage utilisées pour faire le *mapping* des 27 espèces halophiles.

Cheese ID	Cheese Technology	RindType	Country	Animal	Sequencing Technology	Repository	Accession	Reference
1	Soft	Washed	France	Cow	Illumina	SRA	SRR12103075	This study
2	Soft	Washed	France	Cow	Illumina	SRA	SRR12103074	This study
3	Soft	Washed	France	Cow	Illumina	SRA	SRR12103073	This study
4	Soft	Washed	France	Cow	Illumina	SRA	SRR12103072	This study
5	Semihard	Washed	France	Ewe	Illumina	SRA	SRR12103071	This study
6	Soft	Washed	France	Ewe	Illumina	SRA	SRR12103070	This study
7	Blue	Natural	France	Cow	Illumina	SRA	SRR12103069	This study
8	Soft	Washed	France	Cow	Illumina	SRA	SRR12103068	This study
9	Soft	Washed	France	Cow	Illumina	SRA	SRR12103067	This study
10	Soft	Bloomy	France	Goat	Illumina	SRA	SRR12103066	This study
11	Unknown	Natural	USA	Cow	Illumina	MG-RAST	4524482.3	1
12	Unknown	Washed	France	Cow	Illumina	MG-RAST	4524483.3	1
13	Unknown	Bloomy	France	Goat	Illumina	MG-RAST	4524484.3	1
14	Unknown	Bloomy	France	Cow	Illumina	MG-RAST	4524485.3	1
15	Unknown	Washed	Ireland	Cow	Illumina	MG-RAST	4524486.3	1
16	Unknown	Washed	France	Cow	Illumina	MG-RAST	4524487.3	1
17	Unknown	Natural	England	Cow	Illumina	MG-RAST	4524488.3	1
18	Unknown	Bloomy	England	Goat	Illumina	MG-RAST	4524489.3	1
19	Unknown	Natural	USA	Cow	Illumina	MG-RAST	4524490.3	1
20	Unknown	Natural	USA	Cow	Illumina	MG-RAST	4524491.3	1
21	Unknown	Natural	USA	Cow	Illumina	MG-RAST	4524493.3	1
22	Unknown	Washed	USA	Cow	Illumina	MG-RAST	4524494.3	1
23	Unknown	Washed	USA	Cow	Illumina	MG-RAST	4524495.3	1
24	Unknown	Washed	France	Cow	Illumina	MG-RAST	4524496.3	1
25	Unknown	Natural	France	Cow	Illumina	MG-RAST	4524497.3	1
26	Unknown	Natural	Spain	Goat	Illumina	MG-RAST	4524498.3	1
27	Unknown	Washed	USA	Cow	Illumina	MG-RAST	4524499.3	1
28	Unknown	Washed	Switzerland	Cow	Illumina	MG-RAST	4524500.3	1
29	Unknown	Natural	USA	Goat	Illumina	MG-RAST	4524501.3	1
30	Unknown	Washed	France	Cow	Illumina	MG-RAST	4524502.3	1
31	Unknown	Bloomy	Italy	Sheep	Illumina	MG-RAST	4524504.3	1
32	Unknown	Washed	USA	Cow	Illumina	MG-RAST	4524505.3	1
33	Blue	Natural	France	Cow	SOLiD	SRA	SAMEA7202606	This study
34	Blue	Natural	France	Cow	SOLiD	SRA	SAMEA7202608	This study
35	Blue	Natural	France	Cow	SOLiD	SRA	SAMEA7202610	This study
36	Blue	Natural	France	Cow	SOLiD	SRA	SAMEA7202612	This study
37	Blue	Natural	France	Cow	SOLiD	SRA	SAMEA7202614	This study
38	Blue	Natural	Italy	Cow	SOLiD	SRA	SAMEA7202617	This study
39	Blue	Natural	Italy	Cow	SOLiD	SRA	ERS627441	2
40	Blue	Natural	France	Ewe	SOLiD	SRA	SAMEA7202618	This study
41	Soft	Bloomy	France	Cow	SOLiD	SRA	SAMEA7202619	This study
42	Soft	Bloomy	France	Cow	SOLiD	SRA	SAMEA7202620	This study
43	Soft	Bloomy	France	Cow	SOLiD	SRA	SAMEA7202621	This study
44	Soft	Bloomy	France	Cow	SOLiD	SRA	SAMEA7202623	This study
45	Soft	Bloomy	France	Cow	SOLiD	SRA	SAMEA7202625	This study
46	Soft	Bloomy	France	Cow	SOLiD	SRA	SAMEA7202626	This study

Cheese ID	Cheese Technology	RindType	Country	Animal	Sequencing Technology	Repository	Accession	Reference
47	Soft	Bloomy	France	Cow	SOLiD	SRA	SAMEA7202627	This study
48	Soft	Bloomy	France	Goat	SOLiD	SRA	SAMEA7202628	This study
49	Soft	Bloomy	France	Goat	SOLiD	SRA	SAMEA7202629	This study
50	Soft	Washed	France	Cow	SOLiD	SRA	ERS627440	2
51	Soft	Washed	France	Cow	SOLiD	SRA	SAMEA7202631	This study
52	Soft	Washed	France	Cow	SOLiD	SRA	ERS627442	2
53	Hard	Natural	France	Cow	SOLiD	SRA	SAMEA7202632	This study
54	Hard	Natural	France	Cow	SOLiD	SRA	SAMEA7202634	This study
55	Hard	Natural	France	Cow	SOLiD	SRA	SAMEA7202635	This study
56	Hard	Natural	France	Cow	SOLiD	SRA	SAMEA7202636	This study
57	Hard	Natural	France	Cow	SOLiD	SRA	SAMEA7202637	This study
58	Hard	Natural	Italy	Cow	SOLiD	SRA	SAMEA7202638	This study
59	Semihard	Natural	France	Cow	SOLiD	SRA	SAMEA7202639	This study
60	Semihard	Natural	France	Cow	SOLiD	SRA	SAMEA7202640	This study
61	Semihard	Natural	France	Ewe	SOLiD	SRA	SAMEA7202641	This study
62	Semihard	Natural	France	Ewe	SOLiD	SRA	SAMEA7202642	This study
63	Soft	Natural	Spain	Cow	SOLiD	SRA	SAMEA7202644	This study
64	Soft	Natural	France	Cow	SOLiD	SRA	SAMEA7202645	This study
65	Soft	Natural	France	Cow	SOLiD	SRA	SAMEA7202646	This study
66	Soft	Natural	France	Cow	SOLiD	SRA	SAMEA7202647	This study
67	Soft	Natural	France	Cow	SOLiD	SRA	SAMEA7202648	This study
68	Soft	Natural	France	Cow	SOLiD	SRA	SAMEA7202649	This study
69	Soft	Washed	France	Cow	SOLiD	SRA	SAMEA7202650	This study
70	Soft	Washed	France	Cow	SOLiD	SRA	SAMEA7202652	This study
71	Soft	Washed	France	Cow	SOLiD	SRA	SAMEA7202653	This study
72	Semihard	Natural	France	Cow	SOLiD	SRA	SAMEA7202654	This study
73	Semihard	Natural	France	Cow	SOLiD	SRA	SAMEA7202655	This study
74	Semihard	Natural	France	Cow	SOLiD	SRA	SAMEA7202656	This study
75	Soft	Bloomy	Ireland	Cow	Illumina	ENA	ERS3466788	3
76	Soft	Unknown	Ireland	Sheep	Illumina	ENA	ERS3466744	3
77	Soft	Bloomy	Ireland	Cow	Illumina	ENA	ERS3466745	3
78	Semihard	Unknown	Ireland	Cow	Illumina	ENA	ERS3466746	3
79	Semihard	Washed	Ireland	Cow	Illumina	ENA	ERS3466771	3
80	Semihard	Washed	Ireland	Cow	Illumina	ENA	ERS3466747	3
81	Soft	Unknown	Ireland	Cow	Illumina	ENA	ERS3466772	3
82	Soft	Bloomy	Ireland	Cow	Illumina	ENA	ERS3466773	3
83	Hard	Washed	Ireland	Goat	Illumina	ENA	ERS3466748	3
84	Hard	Natural	Ireland	Sheep	Illumina	ENA	ERS3466774	3
85	Semihard	Unknown	Ireland	Cow	Illumina	ENA	ERS3466775	3
86	Soft	Natural	Ireland	Cow	Illumina	ENA	ERS3466749	3
87	Hard	Washed	Ireland	Cow	Illumina	ENA	ERS3466776	3
88	Soft	Washed	Ireland	Cow	Illumina	ENA	ERS3466777	3
89	Soft	Bloomy	Ireland	Cow	Illumina	ENA	ERS3466789	3
90	Soft	Natural	Ireland	Cow	Illumina	ENA	ERS3466790	3
91	Semihard	Bloomy	Ireland	Goat	Illumina	ENA	ERS3466791	3
92	Hard	Natural	Ireland	Cow	Illumina	ENA	ERS3466792	3
93	Soft	Natural	Ireland	Goat	Illumina	ENA	ERS3466793	3
94	Hard	Natural	Ireland	Goat	Illumina	ENA	ERS3466794	3
95	Hard	Natural	Ireland	Cow	Illumina	ENA	ERS3466750	3
96	Hard	Natural	Ireland	Cow	Illumina	ENA	ERS3466751	3
97	Unknown	Natural	USA	Unknown	Illumina	MG-RAST	4675678.3	4
98	Unknown	Natural	France	Unknown	Illumina	MG-RAST	4675679.3	4
99	Unknown	Washed	Spain	Unknown	Illumina	MG-RAST	4675680.3	4
100	Unknown	Natural	USA	Unknown	Illumina	MG-RAST	4675681.3	4
101	Unknown	Washed	USA	Unknown	Illumina	MG-RAST	4675682.3	4
102	Unknown	Natural	Spain	Unknown	Illumina	MG-RAST	4675683.3	4
103	Unknown	Natural	USA	Unknown	Illumina	MG-RAST	4675684.3	4

Cheese ID	Cheese Technology	RindType	Country	Animal	Sequencing Technology	Repository	Accession	Reference
104	Unknown	Natural	England	Unknown	Illumina	MG-RAST	4675685.3	4
105	Unknown	Natural	USA	Unknown	Illumina	MG-RAST	4675686.3	4
106	Unknown	Washed	USA	Unknown	Illumina	MG-RAST	4675687.3	4
107	Unknown	Natural	Italy	Unknown	Illumina	MG-RAST	4675688.3	4
108	Unknown	Washed	France	Unknown	Illumina	MG-RAST	4675689.3	4
109	Unknown	Natural	USA	Unknown	Illumina	MG-RAST	4675690.3	4
110	Unknown	Natural	USA	Unknown	Illumina	MG-RAST	4675691.3	4
111	Unknown	Washed	France	Unknown	Illumina	MG-RAST	4675692.3	4
112	Unknown	Natural	France	Unknown	Illumina	MG-RAST	4675693.3	4
113	Unknown	Natural	USA	Unknown	Illumina	MG-RAST	4675694.3	4
114	Unknown	Natural	France	Unknown	Illumina	MG-RAST	4675695.3	4

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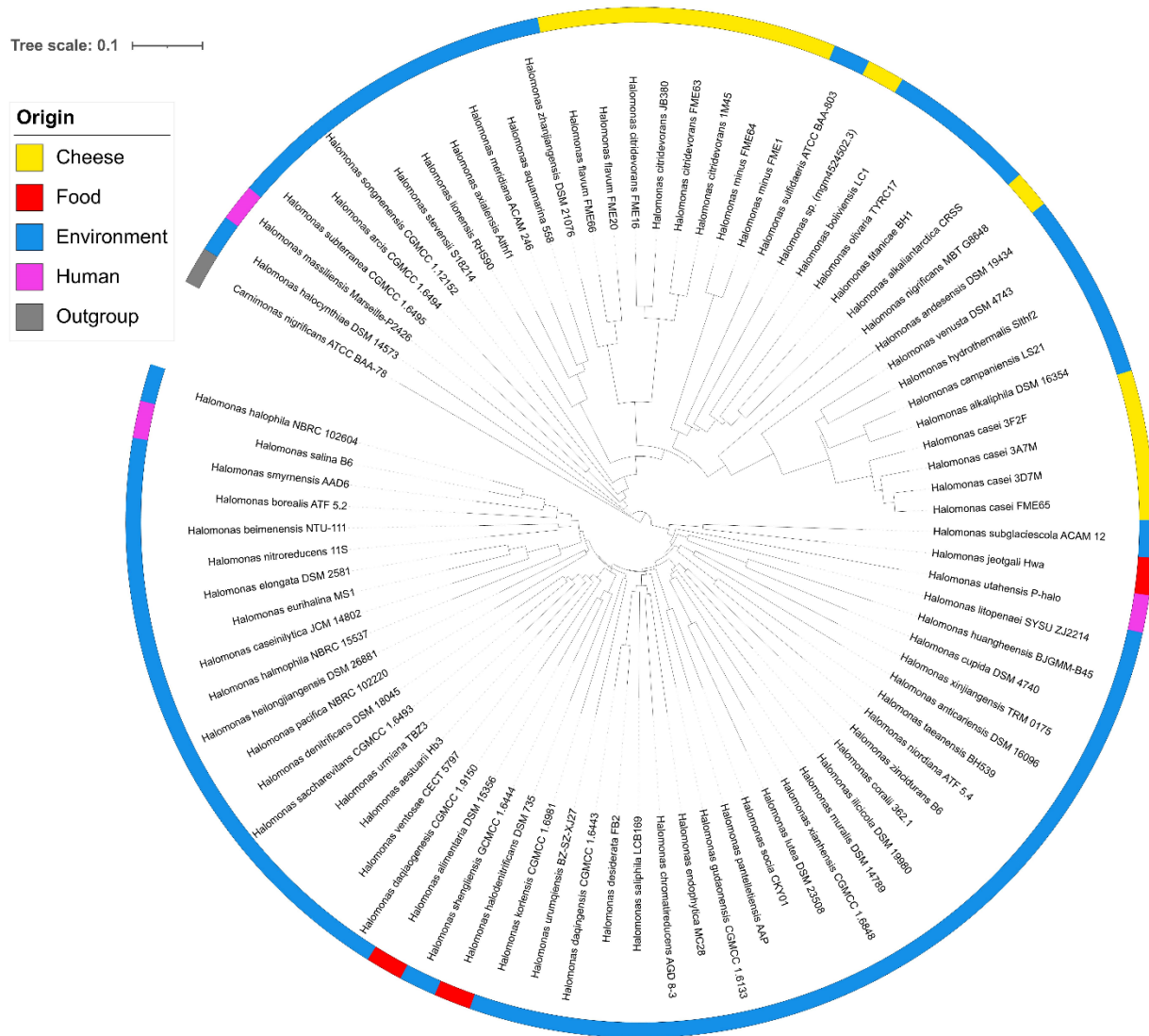
Halomonas

Fig. S1. Topologie phylogénétique pour 82 génomes d'*Halomonas*. Les souches isolées du fromage sont indiquées en jaune, celles d'autres aliments en rouge, celles provenant d'écosystèmes environnementaux en bleu et celles d'origine humaine en rose.

Pseudoalteromonas

Tree scale: 0.1

Origin

- Cheese
- Food
- Environment
- Outgroup

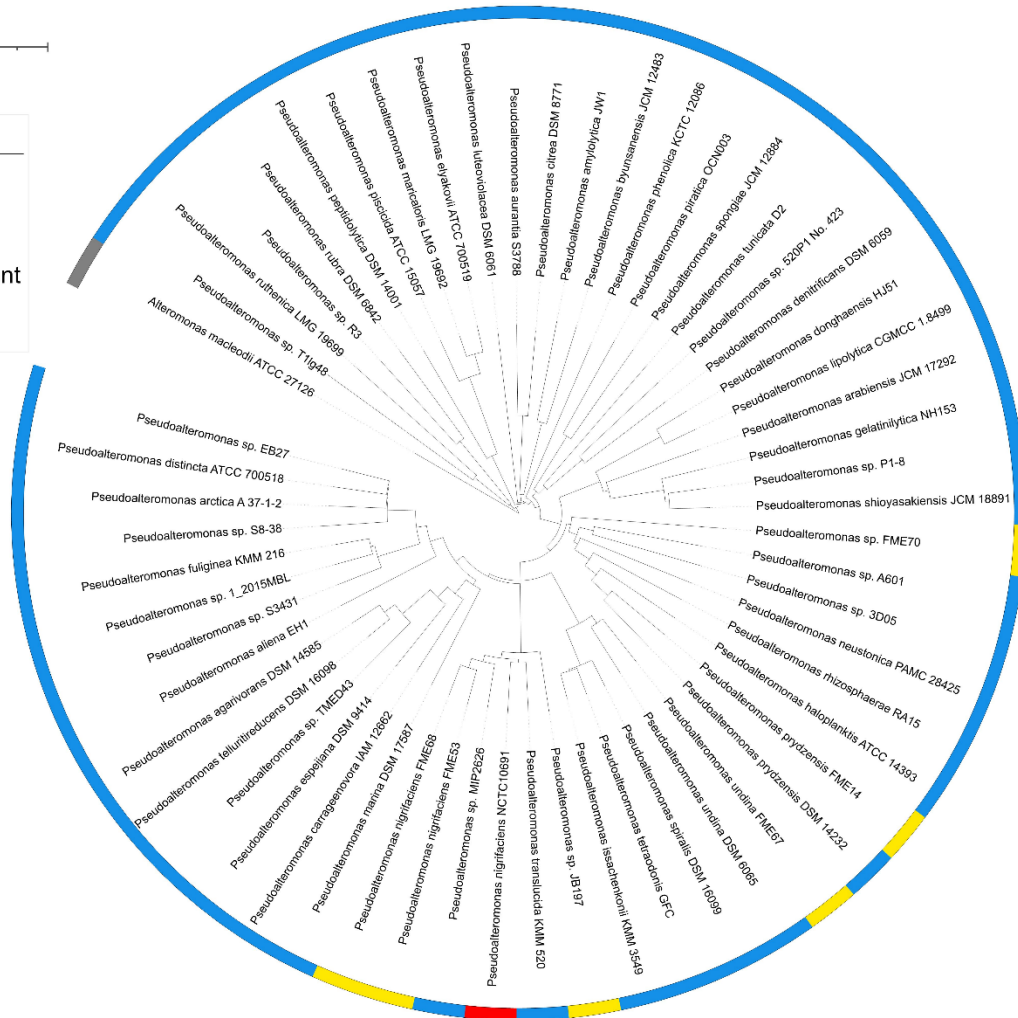


Fig. S3. Topologie phylogénétique pour 58 génomes de *Pseudoalteromonas*. Les souches isolées du fromage sont représentées en jaune, celle d'autre aliment, en rouge et celles des écosystèmes environnementaux, en bleu.

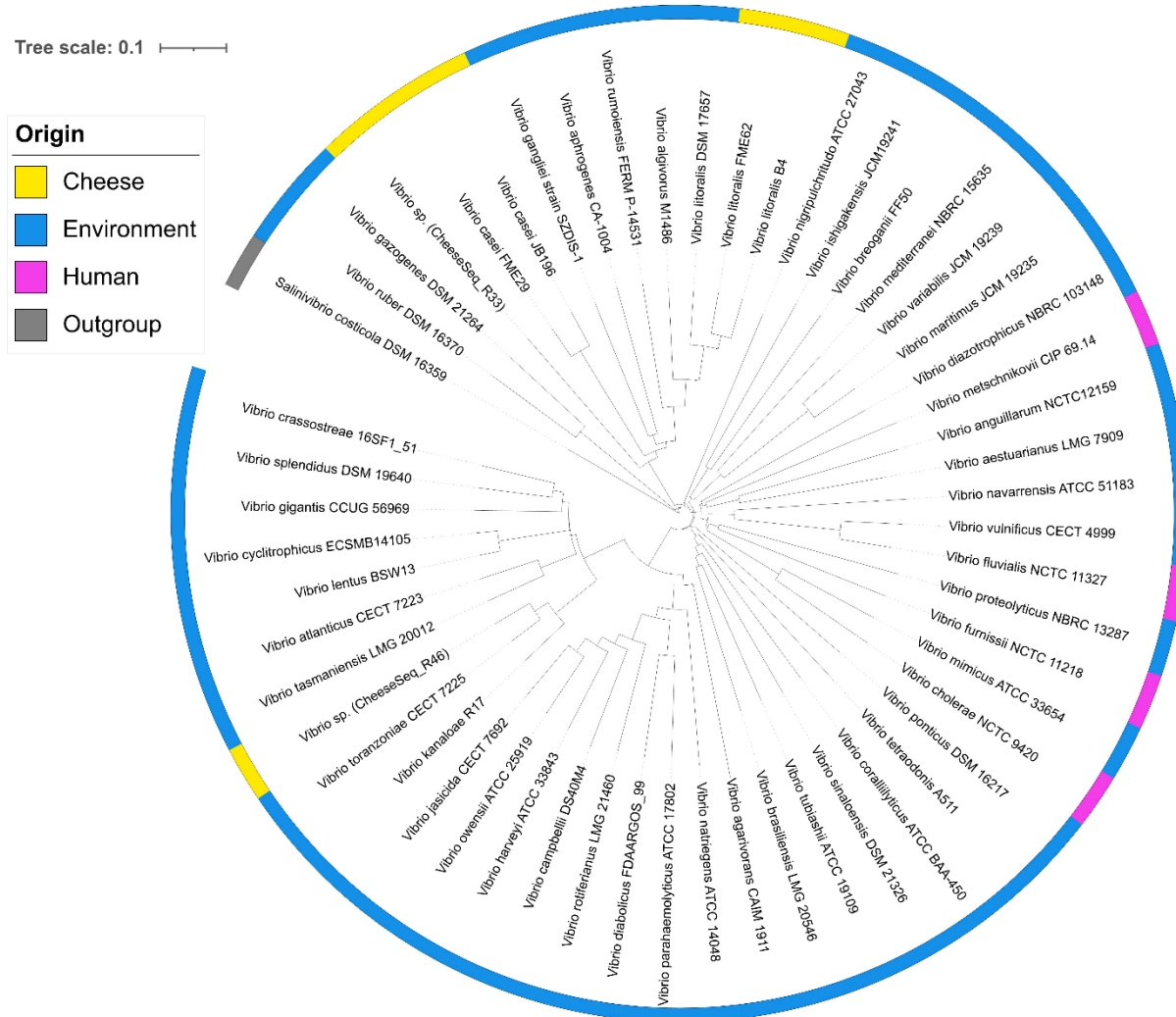
Vibrio

Fig. S5. Topologie phylogénétique pour 54 génomes de *Vibrio*. Les souches isolées du fromage sont indiquées en jaune, celles provenant d'écosystèmes environnementaux, en bleu et celles d'origine humaine, en rose.

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Titre : Diversité des bactéries halophiles dans l'écosystème fromager et étude de leurs impacts fonctionnels

Mots clés : halophiles, halotolérants, génomique, métagénomique, séquençage haut débit, potentiel fonctionnel

Résumé : Les bactéries halophiles et halotolérantes sont généralement isolées dans des environnements naturels, bien qu'elles soient également présentes dans les aliments, comme les croûtes de fromage et les fruits de mer. Cependant, les études sur leurs fonctions écologiques et technologiques dans le fromage sont encore peu développées. Afin de combler le manque de données dans la littérature sur ce sujet, nous avons utilisé des approches culturelles, génomiques et métagénomiques pour déconstruire ces écosystèmes dans les croûtes de fromages. L'approche culturelle, assistée par le développement de stratégies ciblées pour faciliter l'isolement des espèces sous-dominantes, nous a permis d'isoler des représentants de 28 espèces, dont quatre nouvelles espèces d'*Halomonas* et deux de *Psychrobacter*. À partir d'un mapping avec les génomes des espèces halophiles disponibles comme références sur plus de 100 métagénomiques de croûtes de fromages,

nous avons montré que les gamma-protéobactéries, principalement des genres *Halomonas*, *Psychrobacter* et *Pseudoalteromonas*, sont abondantes dans une grande variété de fromages à pâte molle et croûte lavée. Comme ce fait suggère que ces microorganismes pourraient exercer un rôle technologique dans les aliments, nous avons évalué, en parallèle avec des souches issues d'autres environnements, leurs capacités à produire des composés aromatiques et des pigments dans un modèle de fromage synthétique. Ces expériences ont montré que ces gamma-protéobactéries pourraient avoir un impact technologique en plus de leur rôle écologique. Les résultats obtenus dans ce travail devraient stimuler le développement d'études fonctionnelles de cette flore tolérante au sel dans l'écosystème fromager.

Title : Diversity of halophilic bacteria in the cheese ecosystem and the study of their functional impacts

Keywords : halophilic, halotolerant, genomic, metagenomic, high-throughput sequencing, functional potential

Abstract : Halophilic and halotolerant bacteria are usually isolated in natural environments, although they are also present in foods such as cheese rinds and seafood. However, studies on their ecological and technological functions in cheese are still underdeveloped. In order to fill the data gap in the literature on this subject, we have used cultural, genomic and metagenomic approaches to deconstruct these ecosystems in cheese rinds. The cultural approach, assisted by the development of targeted strategies to facilitate the isolation of sub-dominant species, allowed us to isolate representatives of 28 species, including four new species of *Halomonas* and two of *Psychrobacter*. Based on a mapping with the genomes of halophilic species available as reference on more than 100 cheese

rind metagenomes, we showed that gamma-proteobacteria, mainly from the genera *Halomonas*, *Psychrobacter* and *Pseudoalteromonas*, are abundant in a wide variety of soft and washed-rind cheeses. Since this fact suggests that these microorganisms could play a technological role in foods, we evaluated, in parallel with strains from other environments, their ability to produce aromatic compounds and pigments in a synthetic cheese model. These experiments showed that these Gammaproteobacteria could have a technological impact in addition to their ecological role. The results obtained in this work should stimulate the development of functional studies of this salt-tolerant flora in the cheese ecosystem.