

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO	
RAD: 12-164368- -4	FECHA: 2013-09-18 13:29:59
DEP: 2020 DIRECCION DE NUEVAS CREACIONES	EVE: 379 FASE NACIONAL INCLUIDO CAPITULO II
TRA: 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II	FOLIOS: 17
ACT: 519 PRESENTACION	

DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogado(a)/Señor(a)
DANILO ROMERO RAAD

Asunto: Radicación: 12-164368- -4
 Trámite: 11
 Evento: 379
 Actuación: 519
 Oficio: 17798
 Folios: 17

Patente de Invención	X			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	X	Capítulo I		Capítulo II	X
No. Solicitud Internacional	PCT/US2011/025911				
Fecha de Presentación Internacional	23/02/2011				

1. Documentos estudiados

Descripción:	Rad. N° 112-184368--00000-0000	21/Septiembre/2012
	Rad. N° 112-184368--00002-0000	26/Diciembre/2012
Reivindicaciones:	Rad. N° 112-184368--00000-0000	21/Septiembre/2012
	Rad. N° 112-184368--00002-0000	26/Diciembre/2012
Dibujos:	Rad. N° 112-184368--00000-0000	21/Septiembre/2012
	Rad. N° 112-184368--00002-0000	26/Diciembre/2012
Prioridad:	US 61/307,243	23/Febrero/2010

2. Análisis de la Prioridad

Se acepta la reivindicación de prioridad porque ésta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del capítulo primero del Título 10 de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por el solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga más de diez (10) reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las cubiertas por la tasa pagada.

Título de la solicitud: “ADOBOS PARA CARNES Y MARISCOS QUE TIENEN METABOLITOS NATURALES”.

El problema planteado en esta solicitud consiste en la necesidad de adobos que mejoren el rendimiento de la cocción, la jugosidad, el sabor y el color en la carne y los mariscos utilizando únicamente ingredientes naturales.

Para resolver este problema técnico la solicitud en estudio proporciona la composición y aplicación de adobos que contienen, como ingredientes activos claves, una combinación de sales orgánicas y, opcionalmente, péptidos, polipéptidos y fosfolípidos, todos derivados por medio de la fermentación de azúcares.

El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A 23 L 1/31 // 1/314 // 1/315 // 1/318 // 1/39.

4. De la solicitud de patente

Título

El título no corresponde con el objeto de la solicitud porque el término “adobos” es muy general y abarca que contiene muchos compuestos, además no se están mencionando los cultivos bacterianos, por lo tanto se debe especificar al nombre de los componentes, por lo cual se sugiere al Solicitante hacer la corrección necesaria.

Reivindicaciones (Art 30 D 486, Art 22 PCT)

El capítulo reivindicatorio no se encuentra sustentado en la descripción puesto que en las realizaciones preferidas no se evidencia que se parta de cualquier tipo de carbohidrato y que luego este sea sometido a un proceso de fermentación, puesto que en los ejemplos directamente se mencionan las sales de ácidos orgánicos y otros ingredientes como gomas y almidones. Por lo tanto según las realizaciones preferidas el adobo es una combinación de sales de ácidos orgánicos y los llamados “ingredientes funcionales”. Se le aclara al solicitante que las reivindicaciones deben ser claras por si solas para que cualquier persona reproduzca la invención.

La reivindicación 1 no se encuentra debidamente soportadas en la descripción puesto que en las realizaciones preferidas hace referencia aparentemente a la preparación del adobo cuyos componentes están mencionados en la tabla 1, puesto que como tal se mencionan los ingredientes pero no un proceso donde se mezcle y se lleve a cabo la fermentación.

Se sugiere restringir la solicitud a una razonable cantidad de acuerdo al tipo de adobos probados, es decir, que se encuentran debidamente soportados en el capítulo descriptivo. Teniendo en cuenta la descripción en los ejemplos se encuentra:

Tabla 1. Ejemplos del adobo

Componente	Soporte de los ejemplos presentados
Sales de ácidos orgánicos	Lactato, succinato, acetato, propionato, citarto, tartrato
Otros ingredientes funcionales	NaCl, especias, almidones y gomas
bacterias	Especies de <i>proprionibacterium</i>
Bases	NaOH, CaOH

El solicitante debe definir específicamente la materia y el alcance de la invención a través de las características técnicas esenciales que se encuentren enteramente soportadas en la descripción.

La reivindicación 1 no es clara puesto que no se sabe cuántos son los componentes de la formulación, por lo tanto esto debe quedar claramente mencionado en las reivindicaciones.

La reivindicación 1 hace referencia a un adobo pero no se mencionan claramente los componentes y las proporciones de los mismos, por lo tanto se sugiere mencionar cuales son los componentes y sus proporciones. Puesto que hacen parte de las características esenciales de la invención.

En las reivindicaciones 6, 7, 11, 12, 14, 15, 22 y 23 se menciona que la bacteria *propionibacterium* produce los ácidos orgánicos, pero no se menciona que especie de las bacterias *Propionibacterium*, puesto que es un género de bacterias que posee una extensa cantidad de especies. Se sugiere especificar de qué especie se trata, en letra cursiva y anteponer a la palabra *Propionibacterium* el término bacteria.

Las reivindicaciones 1 a 12 y 15 a 16 referentes a un adobo y las 17 a 23 encabezadas como sustrato son las mismas puesto que lo único que las diferencia es el preámbulo de las reivindicaciones, pero tanto lo llamado “adobo” como “sustrato” contienen los mismos ingredientes, solo que en el sustrato si se están mencionando las proporciones. Por lo tanto el solicitante debe definir si lo que quiere reivindicar es un adobo o un sustrato y retirar las reivindicaciones que son iguales.

En las reivindicaciones 1 y 11 no son claras al mencionarse los términos y expresiones “azúcares de la leche”, “azúcares del suero”, “ingredientes conocidos comúnmente encontrados en los adobos”, “ácidos orgánicos”, “fosfolípidos derivados de membranas de células bacterianas”, “péptidos” y “polipéptidos” se están haciendo alusión a unos términos muy amplios, puesto que para una persona de nivel medio versada en la materia, estos evocan su función y no se está indicando la denominación común de los compuestos. Se sugiere definir a qué tipo, tipos u estructura de los anteriores compuestos se está haciendo referencia, pues no se puede definir sólo por su función. De acuerdo a lo sustentado en la descripción.

Las reivindicaciones 1, 2 y 9 no son claras porque mencionan las expresiones “por medio de fermentar azúcares de la leche, azúcares del suero, dextrosa u otros azúcares adecuados...”, “son convertidos a sales orgánicas” y “producidos por medio de fermentar azúcares de la leche, azúcares del suero, dextrosa u otros azúcares...” que corresponden a un resultado a alcanzar, que no definen al adobo porque la invención no queda definida por el resultado a alcanzar, puesto que la reivindicación incluiría no sólo la solución propuesta por el solicitante, sino todas las alternativas presentes o futuras que lleguen a ese resultado. Retirar dichas expresiones.

La reivindicación 7 menciona que se emplean más especies bacterianas, pero es una expresión muy amplia que estaría abarcando infinitudes de especies de bacterias y además dicha expresión no se encuentra sustentado en la descripción puesto que la descripción, en las realizaciones preferidas aparentemente no se emplea ninguna bacteria sino que se nombra que los ácidos son producto de dicha bacteria, pero como tal no se menciona un ejemplo que indique que se agregaron tantas UFC de *propionibacterium*. Por lo tanto agregar otra bacteria no tendría ningún sustento.

El método de las reivindicaciones 13 y 14 no es claro puesto que un método se define por sus características esenciales que consisten en una secuencia de etapas lógica, con las condiciones necesarias para ejecutar cada etapa y claramente en dichas reivindicaciones no se mencionan condiciones de cuantas UFC, de temperatura y tiempo que es de las características básicas de un proceso de fermentación. Además en la llamada etapa de combinar no se menciona las proporciones de cada uno de los componentes que se agrega o adiciona.

5. Determinación del Estado de la Técnica

El Estado de la Técnica se determinó con base en la Fecha de Presentación de la Solicitud de Prioridad, Febrero 23 de 2010 y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D1	XP002632966	"Buttermilk Herb Marinade"	23/Octubre/2006
D2	Giorgio Giraffa, FEMS Microbiology Reviews, vol 28, p 251 – 260	"Studying the dynamics of microbial populations during food fermentation"	2004

El documento D1 ^[1] divulga un adobo para pollo o cerdo y el método de preparación, donde el adobo comprende suero de mantequilla, mostaza Dijon, miel, sal kosher y condimentos: romero, tomillo, salvia, orégano y pimienta negra (Pág. 1).

El documento D2 ^[2] da a conocer que en algunos productos lácteos fermentados, suero de mantequilla cultivado, otras bacterias que se introducen intencionalmente para producir compuestos aromáticos en el suero de mantequilla cultivado (Pág. 253, Col. B, Párr. 2)

6. Examen de Patentabilidad (Art 14 D486)

Nivel Inventivo (Art18 D486)

La **reivindicación 1** consiste en un adobo para carne y mariscos aparentemente definido por sus componentes (Rad. N° 12-164368-000002-00000. Pág. 19)

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D1
Adobo que comprende:	Adobo para pollos y cerdo que comprende:
Ácidos orgánicos	Suero de mantequilla, mostaza Dijon, miel, sal kosher y condimentos: romero, tomillo, salvia, orégano y pimienta negra
Ingredientes comunes de adobo	

El documento D1 es considerado el estado de la técnica cercano a la invención definida en la reivindicación 1 porque divulga un adobo para pollo o cerdo y el método de preparación, donde el adobo comprende suero de mantequilla, mostaza Dijon, miel, sal kosher y condimentos: romero, tomillo, salvia, orégano y pimienta negra (Pág. 1).

La diferencia entre la invención, definida en la reivindicación 1 y el adobo de D1 consiste en que no se menciona directamente la presencia de los ácidos orgánicos, mientras que la solicitud menciona la presencia de los ácidos producto de una fermentación.

El efecto que lograría las diferencias antes mencionada en el adobo de la reivindicación 1, en cuanto mejorar el rendimiento de la cocción, la jugosidad, el sabor y el color, no puede ser determinado toda vez que no existe alguna evidencia que indique que dichas diferencias en cuanto a mencionar la presencia de ácidos orgánicos producto de la fermentación, otorgan al adobo ahora reivindicado una ventaja técnica sobre el adobo ya conocido en la anterioridad D1.

Por lo tanto, el problema técnico objetivo a resolver consiste en plantear una alternativa al adobo conocido según D1 para que se mejore el rendimiento de la cocción, la jugosidad, el sabor y el color.

Sin embargo es conocido en el estado del arte que en la producción del suero de mantequilla cultivado por un cultivo de bacterias lácticas (Pág. 11, Párr. 1) también se

1⁰URL:<http://www.food.com/recipeprint.do?rid=191804>

2⁰Anexo 1, D2

menciona que en las figuras 6.1 y 6.3 se muestra la producción de lactato y acetato a partir de la lactosa, azúcar de la leche, por bacterias ácido lácticas, contiene culturas de bacterias vivas, fosfolípidos y enzimas (Pág. 11, Párr. 1; Pág. 104, Fig. 6.1; Pág. 107, Fig. 6.3; Pág. 111, Fig. 6.8). Además el desarrollo de la acidez por acción bacteriana durante la fermentación de la leche convierte calcio y magnesio a sus formas iónicas. Así el ácido láctico y el acético existen como lactato de calcio o acetato de calcio (Pág. 34, Párr. 3). El pH inicial de la leche es cerca de 6,6 (Pág. 92, Párr.2) y que el citrato de sodio puede ser adicionado al suero de mantequilla para mejorar el sabor, con un máximo legal de 0,15% peso a peso (Pág. 280, Col. b, Párr. 3)³.

Teniendo en cuenta lo expuesto anteriormente, la personas de nivel medio versada en la materia derivara de manera obvia el adobo descrito en la reivindicación 1 de la presente invención a partir de las enseñanzas del adobo del documento D1 y de incluir del estado del arte el hecho que ya es conocido que en la producción de suero de mantequilla se emplea un cultivo de bacterias lácticas y se produce lactato y acetato, por lo tanto las diferencias se limitan en mencionar los ácidos presentes de acuerdo a lo que sucede en las rutas metabólicas produciendo diferentes aromas propios del proceso fermentativo. Por lo tanto al combinar D1 con el estado del arte la reivindicación resulta obvia.

Puesto que las reivindicaciones dependientes 2 – 5, 9 – 10 y 17 – 21 no mencionan características inventivas adicionales, tampoco pueden ser consideradas inventivas.

Conclusión:

Las reivindicaciones 1 – 5, 9 – 10 y 17 – 21, cumplen el requisito de novedad (Art. 16), pero no cumplen con el requisito de nivel inventivo (Art. 18).

La **reivindicación 6** consiste en un adobo para carne y mariscos en el cual el *propionibacterium* es utilizado como cultivo bacteriano (Rad. N° 12-164368-000002-00000. Pág. 19)

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D1	D2
Adobo que comprende:	Adobo para pollos y cerdo que comprende:	Productos lácteos fermentados que comprende:
Ácidos orgánicos	Suero de mantequilla, mostaza Dijon, miel, sal kosher y condimentos: romero, tomillo, salvia, orégano y pimienta negra	Ácidos orgánicos productos de la fermentación
Ingredientes comunes de adobo		No se menciona
<i>Propionibacterium</i> como cultivo bacteriano	No se menciona	<i>Propionibacterium freudenreichii</i>

El documento D1 es considerado el estado de la técnica cercano a la invención definida en la reivindicación 6 porque divulga un adobo para pollo o cerdo y el método de preparación, donde el adobo comprende suero de mantequilla, mostaza Dijon, miel, sal kosher y condimentos: romero, tomillo, salvia, orégano y pimienta negra (Pág. 1).

Además es conocido en el estado del arte que en la producción del suero de mantequilla cultivado por un cultivo de bacterias lácticas (Pág. 11, Párr. 1) también se menciona que en las figuras 6.1 y 6.3 se muestra la producción de lactato y acetato a partir de la lactosa, azúcar de la leche, por bacterias ácido lácticas, contiene culturas de bacterias vivas, fosfolípidos y enzimas (Pág. 11, Párr. 1; Pág. 104, Fig. 6.1; Pág. 107, Fig. 6.3; Pág. 111, Fig. 6.8). Además el desarrollo de la acidez por acción bacteriana durante la fermentación de la leche convierte calcio y magnesio a sus formas iónicas. Así el ácido láctico y el acético existen como lactato de calcio o acetato de calcio (Pág. 34, Párr. 3). El pH inicial de la leche es cerca de 6,6 (Pág. 92, Párr.2) y que el citrato de

³ Ed. Chandan, R.C., et Al. Manufacturing yogurt and fermented milks. Blackwell Publishing. 2006

sodio puede ser adicionado al suero de mantequilla para mejorar el sabor, con un máximo legal de 0,15% peso a peso (Pág. 280, Col. b, Párr. 3) ³.

La diferencia entre la invención, definida en la reivindicación 6 y el adobo de D1 consiste en que no se menciona directamente la presencia del cultivo bacteriano *Propionibacterium*, sino que iría implícito en el suero de mantequilla.

El efecto que lograría las diferencias antes mencionada en el adobo de la reivindicación 5, en cuanto mejorar el rendimiento de la cocción, la jugosidad, el sabor y el color, no puede ser determinado toda vez que no existe alguna evidencia que indique que dichas diferencias en cuanto a mencionar el tipo de cultivo bacteriano empleado, otorgan al adobo ahora reivindicado una ventaja técnica sobre el adobo ya conocido en la anterioridad D1.

Por lo tanto, el problema técnico objetivo a resolver consiste en plantear una alternativa al adobo conocido según D1 para que se mejore el rendimiento de la cocción, la jugosidad, el sabor y el color.

Sin embargo es conocido los diferentes tipos de cultivos bacterianos empleados en los procesos de fermentación, lo cual se menciona en D2 que da a conocer que en algunos productos lácteos fermentados, suero de mantequilla cultivado, otras bacterias tales como *Lactococcus lactis subsp lactis*, *Leuconostoc cremoris*, *Lactobacillus kéfir* y *Propionibacterium freudenreichii* se introducen intencionalmente para producir compuestos aromáticos en el suero de mantequilla cultivado (Pág. 253, Col. B, Párr. 2)

Teniendo en cuenta lo expuesto anteriormente, la personas de nivel medio versada en la materia derivara de manera obvia el adobo descrito en la reivindicación 5 de la presente invención a partir de las enseñanzas del adobo del documento D1 junto con lo conocido en el estado del arte y de incluir el hecho de emplear cultivos de *Propionibacterium* para proporcionar compuestos aromáticos de D2. Por lo tanto al combinar D1 con D2 la reivindicación resulta obvia.

Puesto que las reivindicaciones dependientes 7, 11, 12, 22 y 23 no mencionan características inventivas adicionales, tampoco pueden ser consideradas inventivas.

Conclusión:

Las reivindicaciones 6 a 7, 11, 12, 22 y 23, cumplen el requisito de novedad (Art. 16), pero no cumplen con el requisito de nivel inventivo (Art. 18).

7. Conclusión

Los requisitos de Patentabilidad de la presente Solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	XP002632966	1 – 7, 9 – 12 y 17 – 23	Nivel inventivo
D2	Giorgio Giraffa, FEMS Microbiology Reviews, vol 28, p 251 – 260	6 a 7, 11, 12, 22 y 23	Nivel inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir del día siguiente de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.



JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (c)

El anterior requerimiento fue notificado por fijación en lista, de fecha 2013-09-24

Elaboró: Jhonattan Sanchez Sanchez
Revisó: Gloria Jacqueline Alfonso Roza

Studying the dynamics of microbial populations during food fermentation [☆]

Giorgio Giraffa ^{*}

Istituto Sperimentale Lattiero Caseario, Via Lombardo 11, 26900 Lodi, Italy

Received 31 July 2003; received in revised form 17 September 2003; accepted 20 October 2003

First published online 4 November 2003

Abstract

The dynamics of growth, survival and biochemical activity of microorganisms in food are the result of stress reactions in response to the changing of the physical and chemical conditions into the food microenvironment, the ability to colonise the food matrix and to growth into a spatial heterogeneity, and the in situ cell-to-cell ecological interactions which often happen in a solid phase. In food, ecological approaches to study the evolution of microbial flora would be useful to comprehend better the microbiological processes involved in food processing and ripening, to improve microbiological safety by monitoring in situ pathogenic bacteria, and to evaluate the effective compositions of the microbial populations. This paper gives a general overview of biotechnological approaches to study microbial populations in food fermentation.

© 2003 Federation of European Microbiological Societies. Published by Elsevier B.V. All rights reserved.

Keywords: Food fermentation; Food-associated microorganisms; Microbial dynamics; Culture-independent methods; Intra-specific genetic heterogeneity

Contents

1. Introduction	252
2. Economic importance of food fermentation	252
3. Microorganisms and food fermentation	252
3.1. The starter bacteria	252
3.2. The non-starter bacteria	253
3.3. The unwanted bacteria	253
4. Microorganisms in food ecosystems	254
4.1. Ecological and biochemical factors affecting microbial growth in food	254
4.2. Problems related to qualitative and quantitative estimation of microbial populations in foods	254
4.3. Survival mechanisms of microorganisms in foods	255
5. Monitoring bacterial dynamics in food	255
5.1. Culture-dependent methods	255
5.2. Culture-independent methods	256

[☆] Based on a paper presented at the 1st FEMS Congress of European Microbiologists, Ljubljana (Slovenia), June 29–July 3, 2003.

^{*} Tel.: +39-0371-45011; fax: +39-0371-35579.

E-mail address: ggiraffa@ilclodi.it (G. Giraffa).

6. Concluding remarks	258
References	259

1. Introduction

Food fermentation is the oldest 'biotechnology'. It is responsible for many properties of fermented foods such as flavour, shelf life, texture and health benefits. A variety of fermented foods can be found throughout the world, such as beer, bread, sauerkraut, pickles, cheese, yoghurt, sausages, etc., and their principles offer a wealth of possibilities for new product development. Food fermentation covers a wide range of microbial and enzymatic processing of food and ingredients to achieve desirable characteristics such as prolonged shelf-life, improved safety, attractive flavour, nutritional enrichment, and promotion of health [1].

Fermentation enhances the nutrient content of foods through the biosynthesis of vitamins, essential amino acids and proteins, by improving protein and fibre digestibility, by enhancing micronutrient bioavailability, and by degrading anti-nutritional factors. It also provides a source of calories when used in the conversion into human foods of substrates, which are unsuitable for human consumption. Fermentation processes also enhance food safety by reducing toxic compounds such as aflatoxins and cyanogens, and producing anti-microbial factors such lactic acid, bacteriocins, carbon dioxide, hydrogen peroxide and ethanol which facilitate inhibition or elimination of food-borne pathogens. Therapeutic properties of fermented foods have also been reported. In addition to its nutritive, safety and preservative effects, fermentation enriches the diet through production of a diversity of flavours, textures and aromas. It improves the shelf-life of foods while reducing energy consumption required for their preparation. The production of fermented foods is also important in adding value to agricultural raw materials, thus providing income and generating employment [1,2].

2. Economic importance of food fermentation

The consumption of fermented foods has increased greatly since the 1970s. This includes common foods like dairy products (yoghurt, cheeses, buttermilk), fermented sausages, sourdoughs, fermented alcoholic beverages, vegetables, fruits, and sauces as well as ethnic foods such as kefir and koumiss. One of the reasons for the increase in the consumption of fermented foods is because consumers consider these foods to be healthy and natural. The consumption of

live cells of desirable microorganisms in the billions and of their metabolic products in fermented foods does not cause any panic or distress in safety-concerned consumers [2]. These foods have been around for thousands of years, and therefore have withstood the test of time. A natural next step would be to incorporate the same anti-microbial compounds naturally found in fermented foods into other foods in lieu of "chemical" preservatives.

3. Microorganisms and food fermentation

Within the fermentation industry, microorganisms are used for the production of specific metabolites such as acids, alcohols, enzymes, antibiotics, carbohydrates. Major fermentation microorganisms include lactic acid bacteria (LAB), moulds and yeasts. In particular, LAB are the major microflora involved in fermented dairy products, vegetable, and sourdough fermentation, and (mainly lactobacilli and pediococci) are part of the starter cultures used in meat fermentation to produce desirable acids and flavour compounds. Industrial control of fermentation processes requires up-to-date knowledge of the physiology, metabolism and genetic properties of such microorganisms. Equally important is the knowledge of their impact on food quality, safety and shelf-life. The character of food fermentation, however, is more complex. Agricultural products of animal or vegetable origin are fermented by either the indigenous microflora or an added starter culture to improve or obtain shelf life, nutritional value, health benefit, flavour or texture. Unlike the Western world, Asia has developed many foods based on vegetable proteins using fungi, often in a solid-state fermentation process. In Europe and the US the main focus of food fermentation has been on food preservation by means of acid fermentation, whereas properties such as taste, nutritional value and health effect are more important in Asian (fungi) fermentation. Several authors have written comprehensive reviews about fermented foods [1–5].

3.1. The starter bacteria

In fermentation, the raw materials are converted by microorganisms (bacteria, yeasts and moulds) to products that have acceptable food qualities. Spontaneous (natural) fermentations, i.e., processes initiated without the use of a starter inoculum, have been applied to food

preservation for millennia. In a natural fermentation, the conditions are set so that the desirable microorganisms grow preferentially and produce metabolic by-products, which give the unique characteristics of the product. The majority of small-scale fermentation in developing countries and even some industrial processes such as sauerkraut fermentation are still conducted as spontaneous processes. Various types of starter cultures and even back-slopping are widely used in fermentation processes, even in industrialised countries [1]. However, spontaneous food fermentations are neither predictable nor controllable. The natural microflora of the raw material is either inefficient, uncontrollable, and unpredictable, or is destroyed altogether by the heat treatments given to the food.

When the yield is unstable and where the desired microorganism might not grow, or where pathogenic microorganisms might also grow, a controlled fermentation is used. In a controlled fermentation, the fermentative microorganisms are isolated and characterised, then maintained for use. Since the addition of selected bacteria (usually LAB) starts the manufacturing process, they are commonly referred to as starter cultures. Starter cultures are added to the raw materials in large numbers and incubated under optimal conditions. In common controlled-fermented products such as sauerkraut and yoghurt, the primary function of LAB starters is the production of lactic acid from lactose which helps to make the products shelf-stable. Other functions of starter cultures may include flavour, aroma, and alcohol production, proteolytic and lipolytic activities, and inhibition of undesirable organisms. Therefore, microbial starters provide particular characteristics through fermentation in a more controlled and predictable way.

There are two groups of commercial LAB starter cultures:

- defined (either single or multiple strains): composed of a known number of strains;
- undefined (mixed): in which the number of strains is unknown.

Modern starter cultures are selected either as single or multiple strains, specifically for their adaptation to a substrate or raw material. Suitable cultures for fermentation must be selected at the strain level since not all strains of a species are equally suitable for use as starters, nor are all equally well adapted to a food substrate. In dairy products, the properties of starter cultures such as phage sensitivity, lactic acid, gas, and aroma compounds production are, to a large extent, dependent upon the properties of each culture component [6]. Because modern dairy starter cultures are blended empirically for the desired characteristics of the final product, maintenance of the optimal strain balance throughout the cheese fermentation process is important because the complex relationships among microorganisms can be

easily be altered. Also small variations in microbial composition could have unexpected effects on cheese quality [7].

The largest part of the lactic culture market consists of cultures for the dairy industry. The size of this market is currently approximately US \$250 million [2]. The starter culture industry has been in a phase of consolidation and restructuring; in this context, commercial starter cultures for direct inoculation are increasingly used for their reliability, performance and safety, as well as for their convenience.

3.2. The non-starter bacteria

In some fermented dairy products, additional bacteria such as Cit⁺ *Lactococcus lactis* subsp. *lactis*, *Leuconostoc* spp., *Lactobacillus kefir*, and *Propionibacterium freudenreichii* subsp. *germanii*, often referred to as secondary microflora, are often intentionally introduced to produce aroma compounds and carbon dioxide in cultured buttermilk and certain cheeses. Other types of secondary microflora include undefined mixtures of yeasts (*Debaryomyces* spp. and *Geotrichum* spp.), moulds (*Penicillium camemberti* and *Penicillium roqueforti*), and bacteria (*Brevibacterium linens*, *Micrococcus* spp., and mesophilic lactobacilli). The use of micrococci and *B. linens* is usually limited to surface-ripened and mould-ripened cheeses; mesophilic lactobacilli, which can form biofilms and be a source of contamination in a dairy, are believed to contribute considerably to the formation of cheese aroma from amino acids. For example, a useful cooperation between starter *L. lactis* and glutamate – dehydrogenase-positive *L. casei*/*L. paracasei* strains to stimulate flavour development in Gouda and Cheddar cheeses has been suggested [8].

3.3. The unwanted bacteria

Food manufacturing, distribution, and storage rely on well-placed deleterious stresses or hurdles that either inhibit or inactivate contaminating microorganisms in food systems. However, fermented foods are not commercially produced in an environment free of contaminating microorganisms. For example, dairy products may contain yeasts, moulds, and many different genera of bacteria whose metabolic activities destroy quality. In cheese, after fermentation is complete, only acid-tolerant bacteria can grow. However, if acid development is slow or if the pH does not decrease sufficiently, contaminants which otherwise would have been inhibited may be able to grow. In some fermented foods the pH can increase during ripening and permits the growth of previously inhibited bacteria. These phenomena, together with the well-known evidences that many food-borne pathogenic bacteria are adapting to sub-lethal inimical stresses,

may allow unwanted organisms (either pathogenic or spoilage) to grow in fermented foods.

4. Microorganisms in food ecosystems

The importance of ecological concepts in understanding the growth of microorganisms in foods is well recognised by food microbiologists. These ecological principles are the fundamental to modern quality assurance, predictive modelling, and risk analysis strategies to prevent outbreaks of food spoilage and food-borne disease. They also form the basis for the use of microorganisms in the production of fermented foods and beverages, and for their use as probiotic, starter, and biocontrol agents [9].

4.1. Ecological and biochemical factors affecting microbial growth in food

According to Fleet [9], to effectively manage the growth and activities of microorganisms in fermented foods, the following points should be raised:

- information on diversity, taxonomic identity, growth cycle, quantitative changes, and spatial distribution of microbial species that ferment into the food at every stage of production;
- biochemical and physiological data on the food colonisation process;
- impact of the intrinsic, extrinsic, and processing factors influencing microbial growth, survival and biochemical activity;
- relationship between growth and activity of individual microorganisms and product quality and safety.

4.2. Problems related to qualitative and quantitative estimation of microbial populations in foods

One of the most important applications of ecological concepts to food fermentation ecosystems is the identification, interpretation and manipulation of time-dependent changes in communities of microorganisms. The economic and social consequences of microorganisms in foods depend not only on the species present but, most importantly, on their numbers. The number of microbial cells ultimately determines whether or not the product will cause an outbreak of disease or develop an off-flavour. Unfortunately, the vast majority of ecological studies in food microbiology fall significantly short of providing this quantitative knowledge. Many studies simply describe the isolation and identification of the “most predominant” species at one point in the product’s history, while others have provided semiquantitative data by reporting the frequency of isolation of specific organisms. Population changes are mostly described in reference to microbial groups (e.g., total plate

count, coliforms, LAB) rather than data for particular species or strains, which could be more useful to better comprehend the final microbiological properties of fermented foods.

In a recent and excellent review article, limitations of the methodology to recover microbes from foods, particularly concerning erroneous data given by maceration and dilution steps and quantitative errors coming from underestimation of uncultivable cells, have been summarised [9]. Furthermore, communities of any organisms are static neither in space nor time. This phenomenon, which in ecology is called spatial and temporal heterogeneity, is determined by a sequential development of species and strains occurring in many products, which leads to a continuously changing microbial content, both qualitatively and quantitatively. Heterogeneity results from inherent variability in organic structure and composition of the food (e.g., in surface-smeared cheeses), from changes in the external climatic environment, and as a result of microbial activity.

The dynamics of growth, survival and biochemical activity of microorganisms in foods are the result of stress reactions in response to the changing of the physical and chemical conditions into the food micro-environment (e.g., the gradients of pH, oxygen, water activity, salt, and temperature) and the ability to colonise the food matrix and to grow with spatial heterogeneity (e.g., microcolonies and bio-films). Moreover, because in most food ecosystems microbial populations are assembled to give a community, and because cells are generally immobilised and localised in high densities, food production (or degradation) is rarely the result of the activities of an individual but that of a group of microorganisms [9]. Therefore, the growth, survival, and activity of any one species or strain, whether it be an unwanted spoilage or pathogenic organism, or a desirable biocontrol of a probiotic agent, will, in most cases, be determined by the presence of other microorganisms and the in situ cell-to-cell ecological interactions which often happen in a solid phase. It has been shown that in situ solid phase associations similar to those found in food ecosystems could induce unique biochemical and physiological reactions of a microbial population as a whole [10,11].

Understanding the patterns of occurrence of microbial populations in food is really a problem of determining the actual niche of the organism present; that is, identifying the location (in terms of resource quality, microclimate, and presence of other microorganisms) and the role of an organism in both space and time. Reliable quantitative ecological data should, therefore, take into consideration the dynamics of microorganisms in food ecosystems. This information is of key importance in food ecology, especially to understanding the behaviour of pathogens in foods.

4.3. Survival mechanisms of microorganisms in foods

‘...Microbial ecology is microbial physiology under the worst possible conditions...’ [12]. It is widely accepted that plate culturing techniques reveal a small portion (i.e., that viable and culturable) of the true microbial population in natural ecosystems, and that this can essentially be explained by two ecological factors: (i) an inability to detect novel microorganisms, which might not be cultivable with known media and (ii) an inability to recover known microorganisms which are either stressed or actively growing but which enter a non-cultivable state.

4.3.1. The sub-lethally injured bacteria

Stress is any change in the genome, proteome or environment that imposes either reduced growth or survival potential. Such changes lead to attempts by a cell to restore a pattern of metabolism that either fits it for survival or for faster growth. For any stress, the bacterial cell has a defined range within which the rate of increase of colony forming units (CFUs) is positive (growth), zero (survival) or negative (death). In the first two cases, i.e., growth and survival, the cells are sub-lethally injured, whereas in the case of death (e.g., after a bacteriophage attack) the cells are lethally damaged and rapidly autolyse. The individual values at which the cell moves from one physiological state into the next is conditional on the degree of stress imposed by other environmental conditions [13].

In microbial populations, viable cells are usually countable on both non-selective and selective media, whereas stressed cells are able to form colonies on non-selective media but are not countable on selective media. In foods, many adverse conditions such as nutrient depletion, low temperature and other stresses can sub-lethally damage microorganisms. The viable but non-cultivable (VNC) state is a kind of stress which induces healthy, cultivable cells to enter a phase in which they are still capable of metabolic activity but do not produce colonies on media (both non-selective and selective) that normally support their growth. Sub-lethally injured cells (including VNC cells) are, however, usually able to resume their healthy state [9].

The VNC state has been shown in both Gram positive and Gram negative bacteria in the natural environment and it has also been experimentally induced in most food-borne pathogens [14–16]. Although no molecular technologies have been systematically applied to food ecology, it is conceivable that food-associated microorganisms might enter the VNC state under certain situations, e.g., on the rind of surface-ripened cheese or on the surfaces of fruits and vegetables, where nutrient is limiting. It has been recently suggested that LAB can survive in a VNC state during wine storage and ageing [17].

5. Monitoring bacterial dynamics in food

5.1. Culture-dependent methods

They include traditional cultivation methods in combination with phenotypic (physiological and biochemical) and genotypic (species-specific and RAPD-PCR) identification and typing techniques of isolated strains. Generally, microbiota present during fermentation is enumerated by cultivating on media with the intention of selecting different groups of bacteria (e.g., total aerobic count on PCA medium, presumptive streptococcal and lactococcal counts on LM17 and LM17 plus cycloheximide, respectively, enterococci on KAA medium, leuconostocs on MSE medium, mesophilic lactobacillus on FH medium, thermophilic lactobacillus on MRS medium, etc.). Differences in oxygen tolerance, nutritional requirements, antibiotic susceptibility, and colony morphology and colour constitute the bases of differentiation of these methods. Selectivity of enumeration, however, can be strongly improved by altering incubation temperatures, incubation times, and the pH of the medium. Media allowing the separate (or simultaneous) enumeration of *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Streptococcus thermophilus*, *L. acidophilus*, and *Bifidobacterium* species (such as *B. bifidum* and *B. adolescentis*) are available today for quality control of bioyoghurts [18].

Classical plating methodologies, therefore, only allow a rough measure of microbial groups or genera, and rarely is their sensitivity down to the species level. As stated above, the isolation, identification, and (facultative) typing of many strains from agar plates are needed to increase sensitivity. For the latter two purposes, either phenotyping or genotyping (or both) is applied [19,20]. Concerning phenotyping, identification down to species level can be performed by API galleries or other phenotypic methods such as the *Phene Plate*TM system [21,22]. These techniques allow dynamic trends of cultivable, dominant microbial populations or microbial groups to be highlighted. In recent years, however, genotyping (especially RAPD-PCR) has been successfully applied to identify the presence, succession, and persistence of LAB starter cultures inoculated in silage, sour doughs, probiotic cheese, and meats, as well as to determine the variations of microbial populations in naturally fermented products such as artisan cheeses, olives, meats, sour doughs, and indigenous fermented foods [18,20,23,24]. A variation of this approach is the use of colony hybridisation using group-specific probes to allow specific differentiation and quantification of target population(s) without the need to subculture isolated colonies. Erlandson and Batt [25] described a method using hydrophobic grid membrane filter colony hybridisation for quantitative strain-specific detection of lactococci in bacterial populations. However, classical

plating methods to monitor microorganisms are laborious and time-consuming, especially when both spatial and temporal distribution of microbial populations are needed. Most importantly, culture-dependent methods are not effective if, for any reason, microorganisms are not cultivable.

5.2. Culture-independent methods

To determine the diversity of different microorganisms in natural ecosystems and to monitor evolution of microbial populations over space or time, culture-independent methods have been developed. Compared to traditional culturing, these methods (generally nucleic acids-based) are aimed at obtaining both a qualitative and quantitative picture of a microbial community without the need to isolate and culture its single components. Culture-independent methods have limited applications to food ecosystems.

5.2.1. Applications to foods

Recent applications of DGGE (or TGGE) in identifying major rRNA species present in mixed populations allowed assessment of the impact of probiotic bacteria on the native human gastrointestinal microflora [26–28]. DGGE of PCR-amplified products from the variable region of 16S rRNA genes has been recently used to identify *Lactobacillus* spp. isolated from naturally fermented Italian sausages [29], and to evaluate bacterial diversity occurring in different types of Mozzarella cheese [30]. TGGE was applied to controlled dairy ecosystems with defined compositions, including liquid (starter), semisolid (home-made fermented milk), and solid (miniature cheese models) matrices, and to commercial dairy products. Subspecies, species, or groups of species of LAB were distinguished in dairy samples [31]. LH-PCR was applied to depict population structure of the LAB species recoverable from Grana Padano cheese whey starters (Lazzi, Rossetti, Zago, Neviani, and Giraffa, unpublished data). Typical bacterial species present in the LAB community were well discriminated. Small differences in species composition, e.g., the frequent finding of *S. thermophilus* and the constant presence of thermophilic lactobacilli (*Lactobacillus helveticus*, *L. delbrueckii* subsp. *lactis/bulgaricus*, and *Lactobacillus fermentum*) were reliably highlighted. When present, non-dominant bacterial species present in the whey starters, such as *S. thermophilus* and mesophilic lactobacilli, were easily visualised and characterised without isolating and cultivating single strains. Sensitivity of LH-PCR was confirmed by species-specific PCR from total DNA of the cultures (Lazzi, Rossetti, Zago, Neviani, and Giraffa, unpublished data).

By fluorescence-in situ-hybridisation (FISH) whole fixed cells can be identified and counted directly, i.e., in minimally disturbed samples. In situ identification of

lactococci, enterococci, and streptococci allowed different microbial species in mixed cultures to be distinguished [32]. Specific detection and enumeration of carnobacteria and *Lactobacillus brevis* from sea food products by using ISH flow cytometry has been recently performed [33]. Rapid and sensitive ISH methods have been used for detecting and identifying LAB and brevibacteria in wine and on the surface of Gruyère cheese, respectively [34,35]. In this latter work, it was demonstrated that only a minor part of the microorganisms resident on the cheese surface was cultivable. Millet and Lonvaud-Funel [36] recently found the same behaviour in LAB community enumerated by colony counting and direct epifluorescence technique (DEFT) during wine storage. These latter two examples confirm that classical cultivation techniques are sometimes not able to reveal the most abundant microbes in a given ecosystem. Therefore, the term “dominant” should be used carefully when speaking of food-associated populations in the absence of in situ techniques for microbial counting.

Immunomagnetic separation has been mostly used in the separation of pathogenic bacteria from enrichment cultures and foods [37,38]. FT-IR has been applied as an alternative to the traditional taxonomic techniques to discriminate and identify pathogens, spoilage contaminants, and LAB in foods [39–41].

It should be noted that most of the reported studies have focused on the analysis of end products. DGGE and other techniques have, however, more successfully been applied in polyphasic studies to monitor the microbial dynamics of food ecosystems. By combining different methods (e.g., PCR-DGGE, cloning and sequencing of rDNA amplicons and classical cultivation techniques) it has been possible to profile time-dependent specific changes in the composition of complex microflora, evaluate and quantify non-culturable microbial populations, and among these latter, to monitor the metabolically active microbial groups (or species-groups). This latter point is strongly related to the need to understand the role of unculturable microorganisms in a complex food ecosystem. This can be applied through culture-independent characterisation linked to determination of in situ metabolic activity. Even when an organism can be cultivated, properties determined in the laboratory may not necessarily reflect the activities and physiology of their counterparts in the ecosystem [42,43].

5.2.1.1. Cheese fermentation. DGGE profiles derived from PCR and RT-PCR of DNA and rRNA, respectively, of milk and cheese samples of Ragusano cheese, an Italian artisan cheese produced in Sicily, were compared to determine the expression level of the 16S rRNA genes of the most predominant bacteria, which may reflect their contribution to the total activity [44]. It was possible to: (i) qualitatively profile the dynamics of

prevailing species: in the raw milk (*Leuconostoc mesenteroides*, *Lactococcus lactis*), during lactic fermentation (*S. thermophilus*), and during ripening (*L. delbrueckii*, *L. fermentum*); (ii) evaluate a substantially different degree of metabolic activity for the microbial groups detected, i.e., *L. lactis* and *L. mesenteroides*, both active in milk but not metabolically active in the ripened cheese, and *L. delbrueckii* subsp. *bulgaricus*, which showed an opposite trend; (iii) show a different composition of species identified after cultivation by phenotypic characterisation and those detected by PCR-DGGE, e.g., *L. delbrueckii*, which dominated in the ripening cheese but was not detected by cultivation [44]. The use of RT-PCR is based on the evidence that active bacteria have generally higher numbers of ribosomes than dead or dormant cells. In other words, RT-PCR would provide a rough indication of the relative activities of the major bacteria present. In practice, the low sensitivity of this technology (50 times lower than direct PCR) and the false positivity often detected from non-viable, mild-treated cells (whose ribosomal proteins would protect rRNA from endogenous RNase degradation) underlines the usefulness of RT-PCR in gene expression studies rather than as a cell viability index. In this regard, the application of RT-PCR to messenger-RNA (mRNA) could be more successful because these molecules have a shorter half-life (40 s 20 min) than rRNAs, even in dead cells [45]. By RT-PCR of mRNA it would also be possible to increase our knowledge on gene expression of phenotypic features, especially during food fermentation and manufacturing. In this way, not only the presence of an organism but also some specific metabolic activities of interest could be measured.

A polyphasic PCR-DGGE approach was used to describe the microbial population occurring in natural whey cultures for water-buffalo Mozzarella cheese production [30]. By PCR-DGGE of the 16S rDNA V3 region, the total microbial community was assessed without cultivation by analysing DNA directly extracted from original whey samples and compared to the cultivable community assessed by analysing DNA extracted from combined colonies harvested from different agar plate media. In addition to the four common species evidenced by both total and cultivable community profile (i.e., *S. thermophilus*, *L. lactis*, *L. delbrueckii*, and *L. crispatus*), two additional species (i.e., *L. fermentum* and *E. faecalis*) were highlighted only by the cultivable community. The authors concluded that PCR amplification in the analysis of the total community DNA introduced bias [30].

5.2.1.2. Fermentation of cereal products. Both the qualitative dynamics of the bacterial population and tracing the source of different strains dominating in sourdough fermentation processes using different starter mixtures were shown to be possible by PCR-based methods and

plate culturing [46]. The dynamics of both cultivable and non-cultivable bacteria were monitored by PCR-DGGE and sequencing of partial 16S rDNA genes. Depending on the prevailing conditions in the different sourdough fermentation, only a few *Lactobacillus* species were found to be competitive and became dominant; the results of PCR-DGGE were not always consistent with those obtained by culturing. Isolates of the species *L. sanfranciscensis* and *L. fermentum* were shown by RAPD-PCR analysis to originate from the commercial starters and the baker's yeast, respectively, used to produce the sourdoughs.

A polyphasic approach was applied to evaluate the dynamics of the microbial community responsible for the traditional fermentation of maize in the production of Mexican Pozol [47]. The results confirmed that different culture-dependent or -independent techniques are necessary to obtain a more realistic view of the microbial ecology of this food. In particular, three stages in the microbial dynamics could be clearly detected. Similarly, an integrated strategy using culture-dependent and culture-independent methods was applied to study representative fermenting bacteria from natural cassava dough fermentation [48]. In particular, some *Lactobacillus* species detected in dough samples by sequence analysis of DGGE bands were not recovered under any of the different culture media and conditions used, probably due to the presence of non-culturable strains. On the other hand, some species recovered as pure cultures from enrichments were not detected by direct DGGE analysis of total bacterial DNA from cassava dough, probably because such species were not dominant within the bacterial population.

The development of the LAB community in a commercial malt whisky fermentation was monitored by: (i) estimation of the total bacterial community viability by fluorescence microscopy; (ii) community fingerprinting by PCR-DGGE and DNA sequencing; (iii) indication of the relative activities of the major species present within the LAB community by RT-PCR-DGGE; and (iv) fermentation products accumulation [49]. The evolution of the LAB community occurred in three broad phases. In an early stage yeasts prevailed, whereas LAB were present (especially *S. thermophilus* and heterofermentative lactobacilli) but inhibited; then the exponential growth of lactobacilli belonging to *L. casei* and *L. fermentum* occurred, which was accompanied by notable accumulation of lactic and acetic acids; finally, two new PCR bands were detected on DGGE gels, and the associated bacteria (*L. acidophilus* and *L. delbrueckii*) were largely responsible for the final phases of fermentation [49]. This study suggested that community development in some systems can be thought of as an orderly sequence of chemical changes each catalysed by species waiting their turn in an imaginary eco-queue! Therefore, polyphasic ecology appears helpful in more

predictable analysis of microbial population dynamics in complex, and often unpredictable, food fermentation processes.

6. Concluding remarks

The reported examples underline the possibility of actually monitoring population dynamics in food fermentation, although some basic questions arise, e.g., why are so many food-associated microorganisms in a VNC state? How can we understand their role? What are the environmental factors that lead microorganisms to enter the VNC state in foods? Mechanisms that induce the VNC state have been studied in other microbial environments, e.g., lake water microcosms [50], but little is known about food systems. Methods of studying ecological aspects in food fermentation are very advanced and, once efficiently integrated in a “polyphasic ecology approach”, they could provide the “big picture” of how and why the process is working, and also help in the design and application of starter cultures for the fermented and probiotic food industries. The polyphasic approach, used in particular to study population dynamics in cheese and vegetable fermentation (Table 1) could be applied to other fermented foods, e.g., fermented sausages, and should allow a better understanding of the dynamic changes during traditional (but also industrial) fermentation, with the ultimate objective of controlling the process and the quality of the final food products.

The polyphasic ecology approach, however, does not give an exhaustive answer to more specific food-related

problems arising in the analysis of microbial communities of food ecosystems. For example, how we can increase our knowledge of cell physiology, cell-to-cell interactions, and in situ modification of the microbial metabolism in natural ecosystems, especially in response to adverse environmental conditions; how we can detect and possibly quantify non-culturable and/or non-dominant species/strains; whether there are non-destructive methods of sample preparation in order to better evaluate spatial distribution and colonisation of microorganisms in heterogeneous food matrices. To answer these questions, I reported the possibilities and applications of in situ hybridisation (i.e., FISH) or in situ-PCR. However, FISH carries a number of drawbacks: (i) low sensitivity ($>10^3$ – 10^4 in solid samples). The consequence could be that non-dominant populations are difficult or impossible to enumerate without cell concentration steps. Very recently, however, the sensitivity of FISH has increased considerably, making possible the quantitation of both dominant and non-dominant microbial populations [51,52]. (ii) Methodological limitations (target accessibility, type of fluorochrome, hybridisation conditions) giving highly variable results. (iii) Variability related to the physiological cell state (i.e., slowly growing cells are difficult to detect because of the low rRNA content; damaged and/or stressed cells have variable rRNA content as well).

Another key point to be addressed is that many of the methods reported above are PCR based. PCR is an excellent technique for examining mixed microbial communities. Amplification, however, often introduces biases that make quantification of natural populations difficult. The ability to detect target rRNA sequences

Table 1

The polyphasic strategy applied until now to evaluate microbial content and dynamics in fermented food

Ecological objective	Method(s) applied	Reference(s)
Evaluation of the compositional pattern of the dominant populations	DGGE ^a (or TGGE ^a) + plating	[29–31,44,46,48]
Quantification of non-cultivable populations	FISH ^b (or DEFT ^c) + plating	[35,36,49,52]
Semi-quantification of metabolically active groups	DGGE ^a + RT-PCR ^d ; rRNA quantitative hybridisation	[44,46–49]
Evaluation and monitoring of the fermentation process	Analysis of sugars and fermentation end products accumulation	[47,48]
Detection and quantification of non-dominant species/strains	q-PCR ^e ; c-PCR ^f	[54–57]
Evaluation of the physiological <i>status</i> (viability), stress response, and survival of LAB starters and probiotics in foods	Flow cytometry	[58,59]
(Semi)quantification and dynamics of both dominant and non-dominant microbial populations	Microarray-based rRNA detection without amplification	[53]

^a Denaturing gradient gel electrophoresis (thermal gradient gel electrophoresis).

^b Fluorescence in situ hybridisation.

^c Direct epifluorescence technique.

^d Reverse transcriptase-polymerase chain reaction.

^e Quantitative-polymerase chain reaction.

^f Competitive-polymerase chain reaction.

without amplification (i.e., direct profiling of rRNA sequences) would greatly improve our capacity to determine the relative number of sequences representing natural microbial populations. In this regard, high throughput DNA microarrays, which provide for parallel hybridisations to hundreds or thousands of genes in a single experiment, may play a major role in the study of food systems. In a recent application of oligonucleotide microarrays to environmental ecosystems, the direct profiling of microbial populations was demonstrated by hybridisation of native rRNA recovered from two different sediment samples to a microarray designed to identify major microbial groups [53]. Furthermore, standard PCR reactions are not quantitative. Promising tools for the advancement of studies on food-associated microbial populations, either cultivable or not cultivable, include both competitive-PCR (c-PCR) and quantitative-PCR (q-PCR) [54–57]. Quantitative-PCR has the potential for accurate and highly sensitive enumeration of microorganisms [54]. In one of the few applications of q-PCR to foods, *S. aureus* and *L. monocytogenes* cells in artificially contaminated cheeses and cabbage were quantified with sensitivities of about 10^2 – 10^3 cfu/g [55,56].

Finally, more knowledge is needed on the physiological state (viability), the stress response, and the survival of microorganisms into foods, especially probiotic foods. Stressed, sub-lethally injured, or otherwise “viable but non-culturable” cells often go undetected when using traditional microbiological techniques. In this regard, flow cytometry (FCM) has been recently applied to analyse, in different proportions, sub-populations of variably stressed (or damaged) bacteria in probiotic products and dairy starters [58]. The sensitivity of FCM method was significantly higher than agar plate techniques [59].

References

- [1] Holzapfel, W.H. (2002) Appropriate starter culture technologies for small-scale fermentation in developing countries. *Int. J. Food Microbiol.* 75, 197–212.
- [2] Hansen, E.B. (2002) Commercial bacterial starter cultures for fermented foods of the future. *Int. J. Food Microbiol.* 78, 119–131.
- [3] Wood, B.J.B. (1998) *Microbiology of fermented foods*, Vols. 1–2, second ed. Blackie Academic and Professional, London.
- [4] Caplice, E. and Fitzgerald, G.F. (1999) Food fermentations: role of microorganisms in food production and preservation. *Int. J. Food Microbiol.* 50, 131–149.
- [5] Oyewole, O.B. (1997) Lactic fermented foods in Africa and their benefits. *Food Control* 8, 289–297.
- [6] Wouters, J.T.M., Ayad, E.H.E., Hugenholtz, J. and Smit, S. (2002) Microbes from raw milk for fermented dairy products. *Int. Dairy J.* 12, 91–109.
- [7] Beresford, T.P., Fitzsimons, N.A., Brennan, N.L. and Cogan, T. (2001) Recent advances in cheese microbiology. *Int. Dairy J.* 11, 259–274.
- [8] Kieronczyk, A., Skeie, S., Langsrud, T. and Yvon, M. (2003) Cooperation between *Lactococcus lactis* and nonstarter lactobacilli in the formation of cheese aroma from amino acids. *Appl. Environ. Microbiol.* 69, 734–739.
- [9] Fleet, G.H. (1999) Microorganisms in food ecosystems. *Int. J. Food Microbiol.* 50, 101–117.
- [10] Rehm, H.J., Omar, S.A. (1993) Special morphological and metabolic behaviour of immobilised microorganisms. *Biotechnology*, Vol. 1, second ed., pp. 223–248. Biological Fundamentals, VCH, Weinheim.
- [11] Norton, S. and D'Amore, T. (1994) Physiological effects of yeast cell immobilisation-applications for brewing. *Enz. Microb. Technol.* 16, 365–375.
- [12] Brock, T.D. (1966) *Principles of Microbial Ecology*. Prentice Hall, Englewood Cliffs, NJ.
- [13] Booth, I.R. (2002) Stress and the single cell: intrapopulation diversity is a mechanism to ensure survival upon exposure to stress. *Int. J. Food Microbiol.* 78, 19–30.
- [14] Roszak, D.B. and Colwell, R.P. (1987) Survival strategies of bacteria in the natural environment. *Microbiol. Rev.* 51, 365–379.
- [15] Del Mar Lleo, M., Tafi, M.C. and Canepari, P. (1998) Nonculturable *Enterococcus faecalis* cells are metabolically active and capable of resuming active growth. *System. Appl. Microbiol.* 21, 333–339.
- [16] Del Mar Lleo, M., Tafi, M.C., Signoretto, C., Dal Cero, C. and Canepari, P. (1999) Competitive polymerase chain reaction for quantification of nonculturable *Enterococcus faecalis* cells in lake water. *FEMS Microbiol. Ecol.* 30, 345–353.
- [17] Millet, V. and Lonvaud-Funel, A. (2000) The viable but non-culturable state of wine microorganisms during storage. *Lett. Appl. Microbiol.* 30, 136–141.
- [18] Charteris, W.P., Kelly, P.M., Morelli, L. and Collins, K. (1997) Selective detection, enumeration and identification of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in mixed bacterial populations. *Int. J. Food Microbiol.* 35, 1–27.
- [19] Olive, D.M. and Bean, P. (1999) Principles and applications of methods for DNA-based typing of microbial organisms. *J. Clin. Microbiol.* 37, 1661–1669.
- [20] Giraffa, G. and Neviani, E. (2000) Molecular identification and characterisation of food-associated lactobacilli. *Ital. J. Food Sci.* 12, 403–423.
- [21] Iversen, A., Kühn, I., Franklin, A. and Möllby, R. (2002) High prevalence of vancomycin-resistant enterococci in Swedish sewage. *Appl. Environ. Microbiol.* 68, 2838–2842.
- [22] Lund, B., Adamsson, A. and Edlund, C. (2002) Gastrointestinal transit survival of an *Enterococcus faecium* probiotic strain administered with or without vancomycin. *Int. J. Food Microbiol.* 77, 109–115.
- [23] Gardiner, G., Ross, R.P., Collins, J.K., Fitzgerald, G. and Stanton, C. (1998) Development of a probiotic Cheddar cheese containing human-derived *Lactobacillus paracasei* strains. *Appl. Environ. Microbiol.* 64, 2192–2199.
- [24] Fitzsimons, N.A., Cogan, T.M., Condon, S. and Beresford, T. (2001) Spatial and temporal distribution of non-starter lactic acid bacteria in Cheddar cheese. *J. Appl. Microbiol.* 90, 600–608.
- [25] Erlandson, K. and Batt, C.A. (1997) Strain-specific differentiation of lactococci in mixed starter culture populations using randomly amplified polymorphic DNA-derived probes. *Appl. Environ. Microbiol.* 63, 2702–2707.
- [26] Klaenhammer, T.R. and Kullen, M.J. (1999) Selection and design of probiotics. *Int. J. Food Microbiol.* 50, 45–57.
- [27] Tannock, G.W. (1999) Analysis of the intestinal microflora: a renaissance. *Antonie van Leeuwenhoek* 76, 265–278.
- [28] Walter, J., Tannock, J.W., Tilsala-Timisjarvi, A., Rodtong, S., Losch, D.M., Munro, K. and Alatossava, T. (2000) Detection

- and identification of gastrointestinal *Lactobacillus* species by using denaturing gradient gel electrophoresis and species-specific PCR primers. Appl. Environ. Microbiol. 66, 297–303.
- [29] Cocolin, L., Manzano, M., Cantoni, C. and Comi, G. (2000) Development of a rapid method for the identification of *Lactobacillus* spp. isolated from naturally fermented Italian sausages using a polymerase chain reaction-temperature gradient gel electrophoresis. Lett. Appl. Microbiol. 30, 126–129.
- [30] Ercolini, D., Moschetti, G., Blaiotta, G. and Coppola, S. (2001) The potential of a polyphasic PCR-DGGE approach in evaluating microbial diversity of natural whey cultures for water-buffalo mozzarella cheese production: bias of culture-dependent and culture independent analyses. System. Appl. Microbiol. 24, 610–617.
- [31] Ogier, J.C., Son, O., Gruss, A., Tailliez, P. and Delacroix-Buchet, A. (2002) Identification of the bacterial microflora in dairy products by temporal gradient gel electrophoresis. Appl. Environ. Microbiol. 68, 3691–3701.
- [32] Beimfohr, C., Krause, A., Amman, R., Ludwig, W. and Schleifer, K.H. (1993) In situ identification of lactococci, enterococci and streptococci. System. Appl. Microbiol. 16, 450–456.
- [33] Connil, N., Dousset, X., Onno, B., Pilet, M.F., Breuil, M.F. and Montel, M.C. (1998) Enumeration of *Carnobacterium divergens* V41, *Carnobacterium piscicola* V1 and *Lactobacillus brevis* LB62 by in situ hybridisation-flow cytometry. Lett. Appl. Microbiol. 27, 302–306.
- [34] Sohler, D. and Lonvaud-Funel, A. (1998) Rapid and sensitive in situ hybridization method for detecting, identifying lactic acid bacteria in wine. Food Microbiol. 15, 391–397.
- [35] Kolloffel, B., Meile, L. and Teuber, M. (1999) Analysis of brevbacteria on the surface of Gruyère cheese detected by in situ hybridisation and by colony hybridisation. Lett. Appl. Microbiol. 29, 317–322.
- [36] Millet, V. and Lonvaud-Funel, A. (2000) The viable but non-culturable state of wine microorganisms during storage. Lett. Appl. Microbiol. 30, 136–141.
- [37] Duffy, G., Sheridan, J.J., Hofstra, H., McDowell, D.A. and Blair, I.S. (1997) A comparison of immunomagnetic and surface adhesion immunofluorescent techniques for the rapid detection of *Listeria monocytogenes* and *Listeria innocua* in meat. Lett. Appl. Microbiol. 24, 445–450.
- [38] Ripabelli, G., Sammarco, M.L., Ruberto, A., Iannitto, G. and Grasso, G.M. (1997) Immunomagnetic separation and conventional culture procedure for detection of naturally occurring *Salmonella* in raw pork sausages and chicken meat. Lett. Appl. Microbiol. 24, 493–497.
- [39] Curk, M.C., Peladan, F. and Hubert, J.C. (1994) FTIR spectroscopy for identifying *Lactobacillus* species. FEMS Microbiol. Lett. 123, 241–248.
- [40] Lefier, D., Hirst, D., Holt, C. and Williams, A.G. (1997) Effect of sampling procedure and strain variation in *L. monocytogenes* on the discrimination of species in the genus *Listeria* by FTIR spectroscopy and canonical variate analysis. FEMS Microbiol. Lett. 147, 45–50.
- [41] Lefier, D., Lamprell, H. and Mazerolles, G. (2000) Evolution of *Lactococcus* strains during ripening in Brie cheese using Fourier transform infrared spectroscopy. Lait 80, 247–254.
- [42] Brock, T.D. (1987) The study of microorganisms in situ: progress and problems. Symp. Soc. Gen. Microbiol. 41, 1–17.
- [43] Gray, N.D. and Head, I.M. (2001) Linking genetic identity and function in communities of uncultured bacteria. Environ. Microbiol. 3, 481–492.
- [44] Randazzo, C.L., Torriani, S., Akkermans, A.D.L., de Vos, W. and Vaughan, E.E. (2002) Diversity, dynamics, and activity of bacterial communities during production of an artisanal Sicilian cheese as evaluated by 16S rRNA analysis. Appl. Environ. Microbiol. 68, 1882–1892.
- [45] Sheridan, G.E.C., Masters, C.I., Shallcross, J.A. and Mackey, B.M. (1998) Detection of mRNA by reverse transcription-PCR as an indicator of viability in *Escherichia coli* cells. Appl. Environ. Microbiol. 64, 1313–1318.
- [46] Meroth, C.B., Walter, J., Hertel, C., Brandt, M.J. and Hammes, W. (2003) Monitoring the bacterial population dynamics in sourdough fermentation processes by using PCR-denaturing gradient gel electrophoresis. Appl. Environ. Microbiol. 69, 475–482.
- [47] ben Omar, N. and Ampe, F. (2001) Microbial community dynamics during production of the Mexican fermented maize dough Pozol. Appl. Environ. Microbiol. 66, 3664–3673.
- [48] Miambi, E., Guyot, J.P. and Ampe, F. (2003) Identification, isolation and quantification of representative bacteria from fermented cassava dough using an integrated approach of culture-dependent and culture-independent methods. Int. J. Food Microbiol. 82, 111–120.
- [49] van Beek, S. and Priest, F.G. (2002) Evolution of the lactic acid bacterial community during malt whisky fermentation: a polyphasic study. Appl. Environ. Microbiol. 68, 297–305.
- [50] Signoretto, C., del Mar Lleó, M., Tafi, M.C. and Canepari, P. (2000) Cell wall chemical composition of *Enterococcus faecalis* in the viable but nonculturable state. Appl. Environ. Microbiol. 66, 1953–1959.
- [51] Rudi, K., Nogva, H.K., Moen, B., Nissen, H., Bredholt, S., Mørseth, T., Naterstad, K. and Holck, A. (2002) Development and application of new nucleic acid-based technologies for microbial community analyses in foods. Int. J. Food Microbiol. 78, 171–180.
- [52] Ercolini, D., Hill, P.J. and Hodd, C.E.R. (2003) Bacterial community structure and location in Stilton cheese. Appl. Environ. Microbiol. 69, 3540–3548.
- [53] El Fantroussi, S., Urakawa, H., Bernhard, A.E., Kelly, J.J., Noble, P.A., Smidt, H., Yershov, G.M. and Stahl, D.A. (2003) Direct profiling of environmental microbial populations by thermal dissociation analysis of native rRNAs hybridized to oligonucleotide microarrays. Appl. Environ. Microbiol. 69, 2377–2382.
- [54] Tajima, K., Aminov, R.I., Nagamine, T., Matsui, H., Nakamura, M. and Benno, Y. (2001) Diet-dependent shifts in the bacterial population of the rumen revealed with real time PCR. Appl. Environ. Microbiol. 67, 2766–2774.
- [55] Hein, I., Lehner, A., Rieck, P., Klein, K., Brandl, E. and Wagner, M. (2001) Comparison of different approaches to quantify *Staphylococcus aureus* cells by real-time quantitative PCR and application of this technique for examination of cheese. Appl. Environ. Microbiol. 2001, 3122–3126.
- [56] Hough, A.J., Harbison, S., Savill, M.G., Melton, L.D. and Fletcher, G. (2002) Rapid enumeration of *Listeria monocytogenes* in artificially contaminated cabbage using real-time polymerase chain reaction. J. Food Prot. 65, 1329–1332.
- [57] Felske, A., Akkermans, A.D.L. and de Vos, W. (1998) Quantification of 16S rRNAs in complex bacterial communities by multiple competitive reverse transcription-PCR in temperature gradient gel electrophoresis fingerprints. Appl. Environ. Microbiol. 64, 4581–4587.
- [58] Bunthof, C.J. and Abee, T. (2002) Development of a flow cytometric method to analyze subpopulations of bacteria in probiotic products and dairy starters. Appl. Environ. Microbiol. 68, 2934–2942.
- [59] Budde, B.J. and Rasch, M. (2001) A comparative study on the use of flow cytometry and colony forming units for assessment of the antibacterial effect of bacteriocins. Int. J. Food Microbiol. 63, 65–72.