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ALIMENTACIÓN UNIVERSITARIA: ASPECTOS NUTRICIONALES, MICROBIOLÓGICOS Y TOXICOLÓGICOS.

Mª ISABEL SOSPEDRA LÓPEZ

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Departamento de Medicina Preventiva y Salud Pública, Ciencias de la Alimentación, Toxicología y Medicina Legal

UNIVERSITY FOOD: NUTRITIONAL, MICROBIOLOGICAL AND TOXICOLOGICAL ASPECTS

ALIMENTACIÓN UNIVERSITARIA: ASPECTOS NUTRICIONALES, MICROBIOLÓGICOS Y TOXICOLÓGICOS

Tesis Doctoral Europea

Presentada por:

Mª ISABEL SOSPEDRA LÓPEZ

Dirigida por:

Dr. José Miguel Soriano del Castillo

Dr. Jordi Mañes Vinuesa

Valencia, 2011

VNIVERSITAT DE VALÈNCIA Facultat de Farmàcia
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CERTIFICAN QUE: la licenciada en Farmacia y diplomada en Nutrición Humana y Dietética, Dª Mª Isabel Sospedra López ha realizado, bajo su dirección, el trabajo de investigación titulado: "Alimentación Universitaria: Aspectos Nutricionales, Microbiológicos y Toxicológicos" para optar al grado de Doctor en Farmacia.

Y para que así conste, expiden y firman el presente certificado en Burjassot, a 26 de Septiembre de 2011.

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INDEX

LIST OF ABBREVIATIONS

AESAN: Agencia española de seguridad alimentaria
AGMI: Ácidos grasos monoinsaturados
AGPI: Ácidos grasos poliinsaturados
AGS: Ácidos grasos saturados
APPCC: Análisis de peligros y puntos críticos de control
ARCPC: Análisis de riesgos y control de puntos críticos
ARICPC: Análisis de riesgos e identificación y control de puntos críticos
A_w: Actividad del agua
BP: Baird Parker
BPW: Buffered Peptone Water
CBB: Coomassie Brilliant Blue
CFU: Colony-forming unit
CH_3CN: acetonitrile
CHCA: α-Cyano-4-hydroxycinnamic acid
CT: Cholera Toxin
DAEC: Enteroadherent *Escherichia coli*
DTT: Dithiotreitol
EAEC: Enteroagregative *Escherichia coli*
EDTA: Ethylenediaminetetraacetic acid
EHEC: Enterohemorrhagic *Escherichia coli*
EIEC: Enteroinvasive *Escherichia coli*
ELISA: Enzyme Linked Immunosorbent Assay
EPEC: Enteropathogenic *Escherichia coli*
EPTA: Enfermedades parasitarias transmitidas por alimentos
ETA: Enfermedades transmitidas por alimentos
ETEC: Enterotoxigenic *Escherichia coli*
FBD: Food Borne Disease
H_2O: Water
HACCP: Hazard Analysis and Critical Control Points
HPLC: High Performance Liquid Chromatography
IAA: Iodoacetamide
IDR: Ingestas Dietéticas de Referencia
IL: Interleucina

IMC: Índice de Masa Corporal
ISO: International Organization for standardization
LOD: Limit of detection
LOQ: Limit of quantification
LT: heat labile toxin
LTB: Heat labile toxin subunit B
MALDI-TOF: Matrix-Assisted Laser Desorption/Ionization-Time Of Flight mass spectrometry
MeOH: Methanol
MOWSE: Molecular Weight Search score
MPN: Most Probable Number
NH_4HCO_3: ammonium bicarbonate
PAS: Personal de Administración y Servicios
PCR: Polymerase Chain Reaction
PDI: Personal Docente Investigador
RD: Real Decreto
RPLA: Reverse Phase Latex Aglutination
SA: Sinapinic Acid
SDS-PAGE: **S**odium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEEDO: Sociedad Española para el Estudio de la Obesidad
SEs: Staphylococcal enterotoxins
SST: Síndrome del Shock Tóxico
ST: Stable toxin
TFA: Trifluoroacetic acid
TNF: Factor de necrosis tumoral
TSST-1: Toxic Shock Syndrome Toxin
UCF: Unidades Formadoras de Colonias

INDEX OF TABLES

Tables **Page**

Tables **Page**

Tables **Page**

Tables **Page**

INDEX OF FIGURES

Figures **Page**

SUMMARY

In recent years there has been a significant increase in the consumption of food and beverage in catering services. The current lifestyle of our society has promoted the consumption of food outside home. The catering service at university has become very important in all developed countries in recent decades, being this augment proportional to the increase in university students. Consumers are increasingly demanding healthy and safe food, with better nutritional properties. Meals served must ensure the nutritional quality and limit the exposure to different contaminants that can be found in food. It is therefore important to ensure the quality and variety of menus offered daily, from a nutritional viewpoint as microbiological or toxicological. Meals served must ensure the nutritional quality while limiting the exposure to possible physical, chemical or biological hazards resulting from consumption of meals served at university restaurants.

University students are a vulnerable collective behind these changes. This group has a special interest because many of them assume, for the first time, responsibility for their meal. Dietary habits established by university students when they are away from home, can have a significant effect on their health, determining or modifying the risk of nutrition-related diseases. Several studies have shown that food purchased and/or eaten away from home contribute to inadequate diets. In addition to the nutritional hazards it is desirable to identify, assess and prevent hazards from physical, chemical or biological origin that can affect food safety,

in order to implement appropriate measures to reduce or eliminate hazards to acceptable health standards. University restaurants are catering services, where transformation of food is done to serve customers. The food served must have adequate sanitary and organoleptic quality, it is also important to safeguard the safety, as these foods may be responsible for food poisoning.

Monitoring of polar compounds (legislated in Spain) in oils and fats used is a preventive measure against toxic chemical hazards. These are compounds formed in used oils as a result of changes in fats and oils during frying processes.

The presence of external substances with biological origin in foods can also be cause of foodborne disease (FBD). Products consumed in university establishments can be vectors of several biological contaminants, mainly parasites and bacteria. It is therefore important to have systematic monitoring of the microbiological quality of the food served.

Recently, the prevalence of some parasitic infections, especially human anisakiosis, has increased in many Western countries, including Spain. A large number of marine and freshwater fish can serve as a source of medically important parasitic zoonoses. The most important of the fish-borne helminthes are anisakid nematodes (particularly *Anisakis simplex*).

On the other hand, restaurants must meet certain hygiene standards for production, distribution and trade of ready meals in order to assess the quality and microbiological safety of food. Once the food is ready to eat, microbiological analysis can give us

information about the quality of the process, since the presence of certain microorganisms in foods is a measure of hygiene quality and an indicator of poor handling practices.

The toxicological hazard caused by the presence of microorganisms in food is not represented only by bacteria present in food; but also bacterial toxins in foods eaten are important. Toxins produced by bacteria are proteins and food poisoning symptoms begin within few hours after contaminated food is consumed. Among them deserve particular attention for *E. coli* and *S. aureus* toxins.

E. coli is a bacteria often associated with outbreaks and food poisoning. Under normal conditions, constitutes an essential part of human bacterial flora, however, there are strains capable of causing serious diseases as enteritis. This group of strains is called enterotoxigenic *E. coli* (ETEC) and is transmitted through food or contaminated water by animal or human feces. ETEC labile toxin (LT) is the major virulence factor of ETEC.

S. aureus is the most common toxigenic bacteria in food. It is responsible for most annual cases of food poisoning caused by ingestion of foods where enterotoxins have been preformed. Toxigenic strains of *S. aureus* can produce more than one enterotoxin although enterotoxin type A is the most frequently found in outbreaks, followed by types B and D. *S. aureus* produce also toxic shock syndrome toxin 1 (TSST-1), previously classified as enterotoxin F, which has not been extensively studied in restaurants.

Currently, for enterotoxin detection and isolation have been used different methodologies and, although if some of them are easy techniques or have high sensitivity, their limited specificity or high cost remain as disadvantages. For this reason, the development of rapid and effective techniques for enterotoxin isolation and identification could represent a great advantage for the evaluation of its toxicity and to quantify the amount of toxin produced by strains isolated from food. This will contribute to food security and thereby safeguard consumers´ health in university restaurants.

RESUMEN

Durante los últimos años se ha producido un aumento significativo del consumo de alimentos y bebidas en servicios de restauración. El estilo de vida actual de nuestra sociedad ha impulsado el consumo de alimentos fuera del hogar. La restauración colectiva universitaria ha adquirido una gran importancia en todos los países desarrollados en las últimas décadas, siendo ésta proporcional al aumento de los estudiantes universitarios. Los consumidores demandan cada vez más, alimentos saludables y seguros, con mejores propiedades nutritivas. Por esta razón es importante garantizar la calidad y variedad de los menús ofertados diariamente, tanto desde un aspecto nutricional como microbiológico o toxicológico. Debemos satisfacer las necesidades nutricionales de los consumidores a la vez que se limitan los posibles peligros nutricionales, de origen físico, químico o biológico derivados del consumo de productos en los servicios de restauración universitaria.

Los estudiantes universitarios se presentan como un grupo vulnerable frente a estos cambios, componen un colectivo de interés debido a que muchos de ellos asumen por primera vez la responsabilidad de su comida. Los hábitos alimentarios establecidos por los jóvenes universitarios cuando se encuentran fuera de su hogar pueden tener un efecto importante en su salud, determinando o modificando el riesgo de sufrir enfermedades relacionadas con la nutrición. Existen diversos estudios que demuestran que las comidas hechas fuera de casa contribuyen a dietas poco adecuadas. Además de los peligros nutricionales es

conveniente identificar, valorar y evitar los peligros de origen físico, químico o biológico que pueden afectar a la inocuidad de los alimentos, a fin de poder aplicar las medidas apropiadas para poder minimizarlos hasta niveles sanitariamente aceptables. Los restaurantes universitarios son servicios de restauración colectiva, donde tiene lugar la transformación de los alimentos, mediante procesos de elaboración y prestación de servicios, con el fin de atender a los clientes. Los alimentos servidos deben tener una adecuada calidad organoléptica y sanitaria, para ello es importante cuidar la higiene en los procesos de manipulación, ya que estos alimentos pueden ser responsables de intoxicaciones alimentarias.

El control del nivel de compuestos polares (legislados en España) en aceites y grasas usados es una medida de prevención frente a tóxicos de origen químico. Se trata de compuestos formados en el aceite usado como consecuencia de alteraciones de las grasas y los aceites durante el proceso de fritura.

La presencia de sustancias de origen biológico ajenas al alimento, puede ser también causa de enfermedades trasmitidas por alimentos (ETA). Los productos consumidos en los servicios de restauración universitaria pueden actuar como vectores de contaminantes biológicos, principalmente parásitos y bacterias. Por ello es importante llevar un control sistemático de la calidad microbiológica y parasitológica de los alimentos servidos.

En los últimos años, la prevalencia de la anisakiosis humana, se ha incrementado en numerosos países occidentales, incluida España. Numerosos parásitos infectan al pescado, sin embargo,

sólo unas pocas especies de helmintos son zoonóticas. Las más importantes son los nematodos anisákidos (especialmente *Anisakis simplex*).

Por otro lado, los servicios de restauración deben cumplir determinadas normas de higiene para la elaboración, distribución y comercio de comidas preparadas con la finalidad de determinar la calidad y seguridad microbiológica de los alimentos. Una vez que el alimento está listo para su consumo, su análisis microbiológico puede informarnos acerca del resultado real de todo el proceso, ya que la presencia de determinados microorganismos en los alimentos es una medida de su calidad sanitaria y además un indicador de la incorrección de las manipulaciones efectuadas.

El riesgo toxicológico producido por la presencia de microorganismos en los alimentos no viene representado únicamente por las bacterias presentes sino, en gran medida, por las toxinas bacterianas formadas en los alimentos ingeridos. Las toxinas de las bacterias productoras de intoxicaciones alimentarias se caracterizan por ser proteínas y sus efectos se manifiestan con síntomas agudos a las pocas horas de ser ingeridas. Entre ellas merecen especial atención las de *E. coli* y *S. aureus*.

E. coli es una bacteria frecuentemente asociada con toxiinfecciones e intoxicaciones alimentarias. En condiciones normales, constituye una parte esencial de la flora bacteriana humana, sin embargo, existen cepas capaces de provocar alteraciones graves en forma de enteritis. Este grupo de cepas se denomina *E. coli* enterotoxigénico (ETEC) y se transmiten a través

de los alimentos o el agua contaminada por heces animales o humanas. La toxina termo-lábil (HLT) es su principal factor de virulencia.

S. aureus es la bacteria enterotoxigénica más corriente de los alimentos. Es la responsable de la mayor parte de los casos anuales de intoxicación alimentaria, originada por la ingestión de alimentos en los que se encuentran preformadas enterotoxinas. Las cepas toxigénicas de *S. aureus* pueden producir más de una enterotoxina aunque la enterotoxina del tipo A es la que con más frecuencia aparece en los brotes de intoxicación alimentaria, seguida de los tipos B y D. Merece especial atención la toxina 1 del síndrome del shock tóxico (TSST-1), clasificada antiguamente como enterotoxina F, la cual no ha sido estudiada en profundidad en servicios de restauración.

Actualmente, para la detección y el aislamiento de toxinas bacterianas se utilizan diversas metodologías y, aunque algunas de ellas son sencillas o presentan elevada sensibilidad, su limitada especificidad o el elevado coste continúan siendo una gran desventaja. Por este motivo el desarrollo de técnicas rápidas y selectivas para la identificación de toxinas bacterianas supone una gran ventaja a la hora de evaluar su potencial tóxico y de cuantificar la cantidad producida por cepas aisladas en alimentos, lo que contribuirá a garantizar la seguridad alimentaria y con ello, a salvaguardar la salud de los consumidores en los servicios de restauración universitaria.

LITERATURE REVIEW

1. INTRODUCCIÓN

Los rápidos cambios sociales acontecidos en las últimas décadas han llevado a un importante crecimiento del sector de la restauración colectiva en España y en muchos países occidentales, tanto en el ámbito comercial como en el número de comedores colectivos institucionales o de carácter social (Aranceta *et al.,* 2008; Romero, 2008). A pesar de que las jornadas laborales tienden a reestructurarse de acuerdo con las nuevas situaciones, la comida del mediodía continúa siendo en nuestro país el principal aporte alimentario diario y, en general, se trata de una comida tradicional. La incorporación de las mujeres al mundo laboral y la tendencia a establecer la residencia habitual en los cinturones de las ciudades, a cierta distancia del lugar de trabajo o de estudio, ha desempeñado también un papel clave en esta transformación. Desde el punto de vista de la salud pública, la restauración colectiva universitaria ha adquirido una gran importancia en todos los países desarrollados en las últimas décadas, siendo ésta proporcional al aumento de los estudiantes universitarios. En España, el porcentaje de consumo en estos centros varía dependiendo del tipo titulación impartida, siendo mayor para los grados de las ramas de ciencias, ciencias de la salud y de ingeniería y arquitectura, frente a las de artes y humanidades y ciencias sociales y jurídicas, puesto que las primeras tienen docencia teórica y laboratorio de prácticas lo que abarca todo el

día, imposibilitando, muchas veces, el regresar a casa para comer. Esto se ve reflejado en la tesis doctoral de Riba Sicart (2002) donde el porcentaje de estudiantes de la titulación de Veterinaria en la Universidad Autónoma de Barcelona, que comen en la Universidad, es del 72% frente al 7.9% de los de Psicología.

En nuestro entorno social y cultural, la comida del mediodía es la ración principal del día (Nielsen, 2007). Los aportes realizados en el comedor universitario, por tanto, son especialmente importantes desde los puntos de vista cuantitativo, cualitativo e incluso formativo. El comedor universitario desempeña una importante función alimentaria en cuanto al suministro de alimentos y la composición de los menús ofertados, una función nutricional que permite satisfacer las necesidades nutricionales de los alumnos, del personal docente e investigador y del personal de administración y servicios, una calidad gastronómica y una función educativa, contribuyendo a la construcción de hábitos alimentarios que favorezcan el desarrollo y la promoción de la salud. Para que este planteamiento operativo pueda llevarse a cabo de una manera satisfactoria, es necesario adecuar entre sí los diferentes elementos que lo integran: menú, servicio, utillaje, recinto de cocina y recinto del comedor en torno a las necesidades de los usuarios en el contexto de una propuesta educativa global.

A lo largo de las últimas dos décadas ha aumentado considerablemente la evidencia científica y epidemiológica que sustenta la relación entre distintas características de la dieta y la salud; en unos casos como factor de riesgo y, en otros, como elemento protector (Romaguera *et al.,* 2011). En la actualidad, las

orientaciones en relación con las prácticas alimentarias más saludables y la actividad física constituyen elementos esenciales en todas las estrategias para la prevención de las enfermedades crónicas y de promoción de salud (Woo, 2011). La Organización Mundial de la Salud aprobó en la 57 Asamblea Mundial de Salud en mayo de 2004 la Estrategia Global sobre Dieta, Actividad Física y Salud, justificada por la importancia de favorecer la creación de entornos más saludables (WHO, 2004). Anima, además, a los gobiernos a formular y adoptar políticas que apoyen dietas saludables y limiten la disponibilidad de productos con un alto contenido en sal, azúcares y grasas. Hay que tener en cuenta que los estudiantes universitarios son, en un porcentaje relativamente alto, población cautiva y es de vital importancia garantizar la seguridad y salubridad de los alimentos consumidos, así como asegurar que aporten la energía y los nutrientes necesarios a sus requerimientos en cuanto a su edad, sexo y/o situación fisiológica. Una de las herramientas importantes para conseguir estos objetivos ha sido la introducción de los requisitos de higiene y trazabilidad, así como el sistema de Análisis de Peligros y Puntos Críticos de Control (APPCC) en la industria alimentaria, y en concreto en los servicios de restauración, tras el estudio de Bryan (1990). El sistema APPCC es un sistema relativamente moderno, aplicado por la NASA a finales de los años 60, en los primeros tiempos del programa espacial tripulado de los EEUU, como un sistema para garantizar la salubridad de los alimentos para los astronautas. El sistema fue originalmente diseñado por la Compañía Pillsbury conjuntamente con la NASA y los laboratorios

del ejército de los EEUU en Natick. Esta metodología fue presentada por primera vez, y de forma concisa, en la *National Conference on Food Protection* en 1971. El sistema APPCC ofrece un enfoque sistemático, racional y con base científica para identificar, valorar y evitar los peligros que pueden afectar a la inocuidad de los alimentos, a fin de poder aplicar las medidas apropiadas para poder disminuir o eliminar éstos hasta niveles sanitariamente aceptables. Al dirigir directamente la atención al control de los factores clave que intervienen en la sanidad y calidad en toda la cadena alimentaria, el productor, fabricante y consumidores podrán tener la certeza de que se alcanzan y mantienen los niveles deseados de sanidad y calidad. Con este sistema se desecha el concepto tradicional de inspección del producto final como medio de verificar si nuestro producto es sanitariamente conforme o no. Este sistema, por el contrario, estudia los peligros que pueden presentarse en una determinada industria de forma específica y acorde a las características de la misma, aplicando medidas preventivas que se ajustan al peligro generado, con la ventaja añadida de poder corregir los posibles defectos en proceso, así como modificar y ajustar los controles, evitando así alcanzar etapas posteriores de producción e incluso su consumo. Podemos por tanto definir el sistema de APPCC como un método preventivo que controla de forma lógica, objetiva y sistemática la producción de una industria agroalimentaria (en nuestro caso un establecimiento de restauración colectiva), con el objetivo de producir alimentos sanos e inocuos para el consumidor. Existen diferentes formas de

denominar este programa, como son: Autocontrol Sanitario, ARICPC, ARCPC y APPCC, siendo estas últimas las siglas que nosotros preferimos y que mejor transcriben la terminología con que es conocido internacionalmente, HACCP (*Hazard Analysis and Critical Control Points*), y que se ajusta mejor al concepto y metodología de este sistema. Si se determina que un alimento sea producido, transformado y utilizado de acuerdo con el sistema APPCC, existe un elevado grado de seguridad respecto a su calidad higiénico-sanitaria. El sistema es aplicable a todos los eslabones de la cadena alimentaria, desde la producción, procesado, transporte y comercialización hasta la utilización final en los establecimientos dedicados a la restauración o en los propios hogares. Actualmente esta metodología es de aplicación obligatoria en "todas las empresas con o sin fines lucrativos, ya sean públicas o privadas, que lleven a cabo cualquiera de las actividades siguientes: preparación, fabricación, transformación, envasado, almacenamiento, transporte, distribución, manipulación y venta o suministro de productos alimenticios", según el R.D. 2207/95 que transpone la Directiva 93/43/CE. Sin embargo, conociendo su efectividad contrastada y habiéndose demostrado como el método más eficaz de maximizar la seguridad de los productos, además de otras ventajas como la reducción de costes de no calidad y la optimización de procesos entre otras, sería conveniente su aplicación en todos los eslabones de la cadena alimentaria partiendo del sector productor.

El sistema APPCC en los servicios de restauración se ha aplicado con éxito en España (Soriano *et al.*, 2002a), Estados

Unidos (Althaus, 1995; Chui *et al.*, 2009), Inglaterra (Aruoma, 2006) e Italia (Cenci-Goga *et al.,* 2005; Pontello *et al.,* 2005), incluyendo, en los últimos años, el abordaje planteado sobre cuatro tipos de peligros; nutricionales, físicos, químicos y biológicos (Soriano *et al.,* 2002b).

2. PELIGROS NUTRICIONALES EN LOS SERVICIOS DE RESTAURACIÓN

Originariamente el sistema APPCC abordaba sólo los peligros biológicos, y a lo largo de las dos últimas décadas del siglo XX, se incorporaron los peligros físicos y los químicos. Sin embargo, Soriano *et al.* (2002b) plantearon y justificaron, por primera vez, la posibilidad de añadir en el sistema los peligros nutricionales. Un año más tarde, este grupo de investigación amplió el concepto (Soriano *et al.,* 2003) quedando enfocado tanto a las etapas de procesado que pudieran disminuir o destruir nutrientes como al producto final cuyas cantidades elevadas de nutrientes y/o componentes alimentarios pudieran suponer posibles riesgos para la salud (como es el caso de la presencia de ácidos grasos *trans* o una alta proporción de ácidos grasos saturados). También se incluyó como riesgo nutricional la ingesta elevada de alimentos considerados de consumo ocasional (bollería industrial, bebidas refrescantes y carne grasa), así como la baja ingesta de grupos de alimentos de recomendación diaria (cereales y frutas y verduras entre otras), o incluso aquellas comidas que superaran los consejos sobre la distribución calórica a lo largo del día.

La incorporación de este nuevo peligro ha sido aceptado internacionalmente (Sun and Ockerman, 2005) y su integración en la industria agroalimentaria se llevó a cabo por primera vez en la empresa Nutrimental S. A. (Reissmann, 2005). Rodríguez *et al.* (2010) lo aplicó para el control de la pérdida de vitamina C en verduras y hortalizas en los servicios de restauración, incorporando por primera vez el término "Puntos de Control Nutricional" (*Nutritional Control Points, NCP*) para delimitar las etapas que requieren un control para evitar la pérdida de vitamina C. A su vez, Rafati *et al.* (2010) lo utilizaba en los comedores militares y Rosas and Reyes (2009) en la industria pesquera.

Sobre restauración colectiva universitaria se han realizado diversos estudios (Montero Bravo *et al.,* 2006; González-Osnaya, 2007; Martins Bion *et al.,* 2008). El trabajo de González-Osnaya (2007) fue realizado sobre estudiantes de la Universitat de València y a partir de él se obtuvieron datos sobre el porcentaje de estudiantes consumidores de alimentos en los servicios de restauración universitaria. En un trabajo más reciente (Mendonça *et al.*, 2011) se realizó la valoración nutricional de una muestra de estudiantes de la Universitat de Valencia, así como la determinación de los alimentos más consumidos a lo largo del día.

Durante el curso académico 2009/10 la población universitaria de la Universitat de València estuvo integrada por 59.890, 3.662 y 1.813 alumnos, personal docente e investigador (PID) y personal de administración y servicios (PAS), respectivamente (Universitat de València, 2010). Esto implica que los estudiantes universitarios representan el 91,6% de la población en la Universitat de València,

siendo este colectivo interesante como grupo de estudio debido, no sólo a su representación, sino a que muchos de ellos asumen por primera vez la responsabilidad de su comida.

Durante la realización de éste trabajo (Mendonça *et al.*, 2011) recopilaron datos antropométricos de los estudiantes objeto de estudio. Se tomaron medidas de talla, peso e índice de masa corporal (IMC). Las características antropométricas de la población estudiada aparecen reflejadas en la Tabla 1.

Tabla 1. Valores de la media y desviación estándar de las variables antropométricas por sexos entre deportistas y sedentarios.

	Varones		**Mujeres**	
	Deportistas (n=310)	**Sedentarios (n=110)**	**Deportistas (n=554)**	**Sedentarios (n=325)**
Peso (kg)	76,0 (9,7)	70,8 (6,1)	57,1 (6,9)	65,4 (10,1)
Altura (cm)	175,3 (8,9)	175,1 (6,7)	162,1 (6,7)	163,2 (6,7)
IMC (kg/m^2)	25,1 (3,7)	23,1 (2,8)	21,6 (3,4)*	25,5 (5,4)*

* $p < 0,05$ entre deportistas y sedentarios del mismo sexo.

Los valores de IMC reflejan que un 76,9% de los estudiantes universitarios se encuentran en normopeso. A partir de los datos obtenidos en este estudio se observa que ninguno de los estudiantes participantes en el estudio presentaba obesidad, sin embargo varios de los grupos analizados (hombres deportistas y mujeres sedentarias) superan los límites del normopeso, situándose en el rango de sobrepeso. La Sociedad Española para el Estudio de la Obesidad (SEEDO, 2000), en su documento de consenso establece como puntos de corte en la población adulta

valores de IMC iguales o superiores a 25 kg/m^2 para definir el sobrepeso, y clasifica como sujetos obesos a aquellos que presentan un IMC de 30 kg/m^2 o mayor, contemplando un rango de valores intermedios de riesgo (IMC de 27-29,9 kg/m^2), tipificados como sobrepeso grado II. Según este criterio los varones deportistas son uno de los grupos que presentan sobrepeso. En su caso, los valores de IMC se ven influenciados por el aumento de peso que supone la elevada proporción de masa magra de los participantes deportistas. Estudios precedentes sobre la utilización del IMC como parámetro para valorar el estado y peso ideal de la población concluyen que el IMC no es útil para determinar la composición corporal y por lo tanto, tampoco el peso ideal de deportistas. En el trabajo de Kweitel (2007) queda demostrado que, a pesar de la practicidad del IMC, por ser una herramienta de rápida y sencilla utilización, es poco fiable en el caso de individuos deportistas, ya que llevaría a una incorrecta clasificación de su estado nutricional. En el caso de las mujeres los valores de sobrepeso se han obtenido en el grupo de sedentarias. Teniendo en cuenta que el aporte calórico diario es similar para ambos grupos (incluso menor en mujeres sedentarias) podemos concluir que, de acuerdo a las recomendaciones (FESNAD, 2010), la realización de actividades deportivas contribuye en gran medida a mantener un estado de normopeso.

Mendonça *et al*. (2011) realizó también la valoración dietética de los diferentes grupos de estudiantes, para ello empleó la técnica del "Registro de consumo de alimentos" durante 7 días. El estudiante sujeto de estudio anotó durante una semana todos los

alimentos consumidos cada día. Tras la transformación del consumo de alimentos en energía y nutrientes con el programa informático Easy Diet®, las ingestas diarias de nutrientes se compararon con las ingestas dietéticas de referencia (IDR) para la población española (FESNAD, 2010). En la Tabla 2 se muestran los valores de energía, macronutrientes, fibra y perfil de grasa entre deportistas y sedentarios de ambos sexos. No se encontraron diferencias significativas entre ningún grupo, excepto en el caso de los ácidos grasos poliinsaturados (AGPI) ($p=0,041$) para los varones deportistas y sedentarios. La relación entre los AGPI y los ácidos grasos saturados (AGS) era inferior a las recomendaciones (AGPI/AGS < 0,45), pues la proporción de AGS ingerida es superior a la recomendada. Aranceta y Serra-Majem (2001) recomiendan que la relación de (AGPI+AGMI)/AGS sea > 2. La ingesta energética es notablemente inferior en el caso de las mujeres que en el caso de los varones. Este aporte calórico es deficiente en ambos sexos ya que los valores obtenidos, aunque se aproximan, no alcanzan los recomendados de 2300 y 3000 kcal para mujeres y hombres, respectivamente, para población española que realiza tanto una actividad ligera como moderada.

La media diaria de la ingesta de energía fue de alrededor de 2500 Kcal en hombres deportistas y 2100 en sedentarios. Para las mujeres los valores obtenidos son menores, ambos grupos ingieren una media de 1900 Kcal diarias sin diferencia significativa entre ellos. Sin embargo, si que se observa esta diferencia entre géneros.

Tabla 2. Valores de la media y desvío estándar de la energía, macronutrientes, fibra, perfil de grasa y agua.

	Varones		Mujeres	
	Deportistas (n=310)	**Sedentarios (n=110)**	**Deportistas (n=554)**	**Sedentarios (n=325)**
Energía				
Por día (kcal)	2575±701	2135±327	1940±716	1899±384
Por día (KJ)	10765±2931	8924±1368	8111±2993	7938±1605
Unidad por kg (KJ/kg)	147,9±35,5	123,4±36,7	141,5±65,2	125,1±33,5
Proteína				
Por día (g)	99,4±29,9	86,6±12,6	79,2±30,9	74,7±19,0
Unidad por kg (g/kg)	1,4±0,4	1,2±0,3	1,4±0,7	1,2±0,4
Proporción de energía (%)	15,4±4,1	16,3±3,8	16,3±2,9	15,7±3,1
Grasa				
Por día (g)	114,5±35,2	102,5±26,3	90,7±42,4	88,3±20,4
Unidad por kg (g/kg)	1,6±0,4	1,4±0,6	1,6±0,9	1,4±0,4
Proporción de energía (%)	43,2±2,6	46,8±2,4	44,1±1,8	44,2±2,6
Carbohidratos				
Por día (g)	266,2±82,1	196,3±36,4	192,4±58,4	190,2±51,2
Unidad por kg (g/kg)	3,7±1,0	2,7±0,8	3,3±1,3	2,9±0,9
Proporción de energía (%)	41,2±7,9	36,8±9,4	39,6±9,8	40,1±10,4
Fibra				
Por día (g)	26,5±27,2	15,8±2,9	15,9±4,5	15,1±4,0
AGMI				
Por día (g)	50,3±16,3	48,8±9,5	41,1±17,6	39,7±10,6
Proporción de energía (%)	7,8±1,5	9,2±1,6	8,5±1,5	8,5±1,9
AGPI				
Por día (g)	15,3±5,9*	10,5±0,8*	10,2±4,6	9,8±2,4
Proporción de energía (%)	2,4±0,6*	2,0±0,2*	2,1±0,5	2,1±0,5
AGS				
Por día (g)	38,9±13,6	32,8±14,2	31,1±17,8	31,4±9,3
Proporción de energía (%)	6,0±1,2	6,0±1,7	6,2±1,0	6,5±1,0
AGPI / AGS	0,41±0,1	0,36±0,1	0,34±0,1	0,32±0,1
(AGPI + AGMI) / AGS	1,7±0,4	2,0±0,7	1,8±0,4	1,6±0,4
Colesterol (mg)	412,6±162,9	394,3±98,3	378,7±221,7	336,6±119,6
Agua (ml)	2120±354,2	1925±369,4	2002±401,4	1875±247,5

* $p < 0,05$ entre deportistas y sedentarios del mismo sexo.

Los cuatro grupos estudiados presentan un porcentaje alto de ingesta de proteínas y grasas. Sin embargo, el porcentaje de hidratos de carbono es porcentualmente bajo con respecto a las calorías totales establecidas por los Objetivos Nutricionales para la población española (30-35 % de lípidos, 50-55 % de hidratos de carbono y entre el 10-15 % de proteínas) Aranceta *et al.* (2001). Estos datos se reflejan en otros estudios de población universitaria (González *et al.,* 1999).

En cuanto a los datos de colesterol obtenidos por Mendonça *et al*. (2011), el consumo medio para los dos sexos es superior al máximo permitido (300 mg/día) Aranceta *et al.* (2001), siendo en el caso de los varones deportistas (412,6 mg/día) superior al de las mujeres sedentarias (336,6 mg/día).

En el caso de la fibra el consumo en ambos sexos es inferior al recomendado, excepto en el caso de varones deportistas (26,5 g/día) (Mendonça *et al*., 2011). Esta tendencia a un bajo consumo se repite no sólo en este estudio sino en otros como es el caso en la Universidad de Granada (López *et al.,* 2006), Universidad Alfonso X el Sabio de Madrid (Martínez Roldán *et al.,* 2005), Universitat de València (Miere *et al.,* 2007; González-Osnaya, 2007; Soriano *et al.,* 2000a), Universidad San Pablo-CEU (Oliveras *et al.,* 2006) y universitarios de Galicia (Díaz Mejía *et al.,* 2005) cuyos valores medios son de 9,8, 15,3-18,1, 17,7-19,5, 15,1-16,4 y 14,5 g/día, respectivamente.

Con respecto a la ingesta vitamínica (Tabla 3), se observan diferencias significativas (p=0,047) principalmente entre hombres deportistas y sedentarios (Mendonça *et al*., 2011). Según las

ingestas dietéticas de referencia, los varones deportistas solamente presentan un déficit de vitamina E. En los varones sedentarios, sin embargo, el déficit es de vitamina A, D y E, folato y biotina. Esto se refleja en los datos del Libro Blanco de las vitaminas en la alimentación de los españoles (Aranceta *et al.,* 2000) y en otros estudiados realizados tanto en España (Turnes Carou *et al*., 2001; Serra-Majem *et al*., 2002) como fuera de España (Samuelson *et al.,* 1996; Andersen *et al.,* 1995; Johansson *et al.,* 1997; Koening and Elmadfa, 2000). Las mujeres de la Universitat de Valencia que participaron en el estudio de Mendonça *et al*. (2011), tanto deportistas como sedentarias, tienen una baja ingesta de vitamina D y E, folato y biotina.

En la Tabla 3, se observan también las ingestas de minerales. Encontramos diferencias significativas (p=0,038) principalmente entre hombres deportistas y sedentarios.

Según las ingestas dietéticas de referencia (FESNAD, 2010), persiste un elevado consumo de sodio y un déficit de potasio, calcio, yodo y magnesio en todos los grupos de estudiantes, siendo estos valores similares a los obtenidos por otros autores en España (Lage Yusty *et al.,* 1999; García Segovia and Matínez Monzó*,* 2001; González Castro *et al.,* 2001; 336 González-Carnero *et al.,* 2002a, 2002b). Además, los resultados coinciden con los obtenidos en otros países europeos (Welten *et al.,* 1996; Andersen *et al.,* 1997; Johansson *et al.,* 1997; Evans and Dowler, 1999; Van de Vijver *et al.,* 1999; Ysart *et al.,* 1999; Koening and Elmadfa, 2000), americanos (Mailhot *et al.,* 1994; Schieri and Everhart, 1998) y asiáticos (Du *et al.,* 2000).

Tabla 3. Valores de media (X) y desvío estándar (DE) de la ingesta diaria de vitaminas y minerales por sexos entre deportistas y sedentarios.

	Varones				Mujeres			
	Deportistas (n=310)		Sedentarios (n=110)		Deportistas (n=554)		Sedentarios (n=325)	
	X±DE	% Ingesta real respecto a las IDR	X±DE	% Ingesta real respecto a las IDR	X±DE	% Ingesta real respecto a las IDR	X±DE	% Ingesta real respecto a las IDR
Tiamina (mg)	2,0±1,0*	166,7	1,4±0,3*	116,7	1,4±0,7	140,0	1,3±0,6	130,0
Riboflavina (mg)	2,9±1,7*	181,2	1,7±0,4*	106,2	1,8±0,7	138,5	1,8±0,6	138,5
Niacina (mg)	48,0±18,5*	266,7	38,7±5,0*	215,0	34,0±11,6	242,8	33,0±8,8	235,7
Ácido pantoténico (mg)	7,2±2,9*	144,0	5,2±0,8*	104,0	5,2±2,0	104,0	5,5±2,0	110,0
Vitamina B6(mg)	3,2±1,5*	213,0	2,3±0,5*	153,3	2,0±0,8	166,7	2,1±0,8	175,0
Biotina (µg)	36,1±22,7*	120,3	26,9±7,1*	96,7	27,6±12,8	92,0	26,6±12,4	88,7
Folato (µg)	342,2±166,7*	114,1	213,8±47,7*	71,3	223,4±86,6	74,5	230,8±84,5	76,9
Vitamina B12(µg)	6,5±3,3	325,0	5,7±1,3	285,0	4,6±1,7	230,0	5,0±3,2	250,0
Vitamina C (mg)	160,0±100,1*	266,7	86,5±25,6*	144,2	123,7±54,9	206,2	104,4±52,1	174,0
Vitamina A (µg)	1149±712,1	164,2	629±405	89,8	845,2±401,2	140,9	942,9±395,5	157,2
Vitamina D (µg)	5,6±4,0	112,0	4,6±3,4	92,0	2,6±1,5	52,0	3,8±3,2	76,0
Vitamina E (mg)	9,0±3,4*	60,0	6,9±0,7*	46,0	7,2±2,7	48,0	6,4±1,8	42,7
Sodio (mg)	2629±882,2	175,3	2536±577	169,0	2321±1065	154,8	2362±1155	157,4
Potasio (mg)	3594±1362*	115,9	2533±463*	81,7	2777±1082	89,6	2608±791,2	84,1
Calcio (mg)	1083±465,8*	120,4	852,3±220,1*	94,7	891,6±346,9	99.0	812,9±265,7	90,3
Magnesio (mg)	350,0±123,7*	100,01	256,5±40,6*	73,3	252,9±91,8	84,3	242,3±59,3	80,8
Fósforo (mg)	1730±547,9*	247,1	1381±104,1*	197,3	1337±444,1	190,9	1277±348,3	182,4
Hierro (mg)	21,1±12,9*	234,4	11,8±2*	131,1	12,8±4,9	71,1	13,1±5,2	72,8
Zinc (mg)	11,6±4,2*	122,1	9,7±0,7*	102,1	9,4±3,9	134,3	8,5±2,4	121,4
Selenio (mg)	133,6±46,6*	242,9	109,7±27,3*	199,4	101,7±22,8	184,9	92,6±24,0	168,4
Yodo (µg)	117,4±42,2*	78,3	107,3±9,1*	71,5	92±29,4	61,3	88,4±31,3	58,9

* $p < 0,05$ entre deportistas y sedentarios del mismo sexo.

Tabla 4. Valores de media y desviación estándar de la ingesta diaria de energía por grupos de alimentos por sexos entre deportistas y sedentarios.

	Varones		Mujeres	
	Deportistas (n=310)	**Sedentarios (n=110)**	**Deportistas (n=554)**	**Sedentarios (n=325)**
Cereales y derivados				
Energía (kcal)	802,6±237,8	622,8±232,2	597,1±184,4	634,3±250,9
Legumbres				
Energía (kcal)	43,0±52,0*	21,1±42,2*	17,6±35,0	18,7±32,3
Verduras y Hortalizas				
Energía (kcal)	97,9±46,8	91,2±27,4	90,8±33,5	90,3±49,3
Frutas				
Energía (kcal)	130,1±115,9*	80,8±22,1*	115,1±67,2*	68,3±51,6*
Lácteos y derivados				
Energía (kcal)	318,1±170,8	259,3±126,4	296,9±150,5	254,6±127,6
Carnes y derivados				
Energía (kcal)	287,0±154,0	283,0±43,7	232,6±198,5	222,0±132,3
Pescados y derivados				
Energía (kcal)	79,4±63,7	104,4±63,0	58,7±44,6	61,6±52,5
Huevos y derivados				
Energía (kcal)	56,0±53,2	51,0±15,5	52,4±41,8	45,7±37,8
Azúcares, dulces y pastelería				
Energía (kcal)	150,5±243,1	90,6±72,5	78,2±82,7	105,8±117,8
Aceites y grasas				
Energía (kcal)	408,2±177,8	419,0±144,7	307,4±146,8	288,1±141,5
Bebidas				
Energía (kcal)	146,4±162,4	76,4±116,7	49,7±68,3	66,6±70,1
Platos preparados y precocinados				
Energía (kcal)	0,8±1,1	0,0±0,0	2,1±3,3*	7,5±20,5*
Aperitivos				
Energía (kcal)	15,2±33,4*	31,7±36,9*	31,9±52,9*	19,0±33,5*
Salsas y condimentos				
Energía (kcal)	40,2±50,5*	3,6±1,3*	10,4±13,8	16,9±36,8

*p < 0,05 entre deportistas y sedentarios del mismo sexo.

Según éste trabajo (Mendonça *et al.*, 2011), sólo las mujeres muestran déficit de hierro. De hecho, hay que tener en cuenta que el déficit de hierro es uno de los problemas nutricionales más comunes en el mundo, presentando aproximadamente el 40% de la población mundial anemia o deficiencia de hierro (Du *et al.,* 2000). Por lo que se refiere concretamente a la población joven del mundo desarrollado, aproximadamente el 20% de las mujeres en edad fértil presentan deficiencia de este mineral.

Con los valores obtenidos sobre la ingesta de alimentos de los estudiantes de la Universitat de València quedan patentes los peligros nutricionales en lo referente al consumo diario de macro y micro nutrientes, evidenciándose que existen ciertos desequilibrios en macronutrientes (altos en ingesta de grasas y proteínas y bajas en carbohidratos) y micronutrientes (diez de los veintiun parámetros presentan valores inferiores a las IDR para la población española).

Los cereales y derivados se destacaron como el grupo más consumido diariamente por un mayor porcentaje de estudiantes, seguido de los aceites y las grasas, lácteos y derivados, carnes y productos cárnicos, azúcares, dulces y pastelería, frutas, verduras y hortalizas, pescados y derivados, bebidas, legumbres y huevos. Siendo los aperitivos, junto con los platos precocinados y las salsas y condimentos, los grupos que menor porcentaje calórico aportan a la dieta de los estudiantes universitarios.

Los resultados obtenidos por este grupo están en concordancia con los estudios de Miere *et al.*, 2007; González-Osnaya, 2007; Soriano *et al.*, 2000a, Oliveras *et al.*, 2006, dónde se

refleja este hecho como la situación normal entre los estudiantes universitarios valencianos y madrileños. El conocimiento de los alimentos más consumidos en los servicios de restauración de la Universitat de València será útil para realizar un análisis microbiológico completo y representativo de los platos preparados servidos en los restaurantes universitarios.

3. PELIGROS FÍSICOS EN LOS SERVICIOS DE RESTAURACIÓN

Los peligros físicos son a menudo descritos como materia extraña u objetos ajenos e incluye cualquier material que normalmente no se encuentra en el alimento el cual puede causar enfermedades (incluyendo traumas psicológicos) o heridas a un individuo (Zakocs *et al.,* 1998). En la Tabla 5 se presentan los materiales más importantes que se consideran como peligro físico y las fuentes más comunes (Bryan, 1990).

Tabla 5. Principales materiales de importancia como peligro físico y fuentes comunes.

MATERIAL	DAÑO POTENCIAL	FUENTES
Vidrio	Corte, hemorragia	Botellas, jarras, artefactos lumínicos, utensilios, etc
Madera	Corte, infección, asfixia	Cajas, pallets, campos, estructuras de madera
Piedras	Asfixia, rotura dentaria	Edificio, campos

Metal	Corte, infección	Maquinarias, campos, cables, empleados
Insectos y otras suciedades	Enfermedad, trauma, asfixia	Campo, entrada de planta post-proceso
Aislantes	Asfixia, asfixia por asbestos (largo plazo)	Materiales del edificio
Huesos	Asfixia, trauma	Campo, procesado impropio de la planta
Plástico	Asfixia, cortes, infección	Campos, materiales de envasado, pallets
Efectos personales	Asfixia, cortes, rotura dentaria	Empleados

Actualmente, no existe ninguna referencia bibliográfica sobre la presencia de peligros físicos en servicios de restauración.

4. PELIGROS QUÍMICOS EN LOS SERVICIOS DE RESTAURACIÓN

El peligro químico es aquel riesgo susceptible de ser producido por una exposición no controlada a agentes químicos la cual puede producir efectos agudos o crónicos y la aparición de enfermedades. Los productos químicos tóxicos también pueden provocar consecuencias locales y sistémicas según la naturaleza del producto y la vía de exposición. Todos los productos alimenticios poseen químicos y todos los químicos pueden ser tóxicos a cierto nivel de concentración. Sin embargo, un número de químicos no está permitido en los alimentos y otros tienen establecidos límites. En la Tabla 6 se muestra una recopilación de los peligros químicos divididos en cuatro grupos: Muchos de ellos pueden potencialmente causar intoxicaciones químicas si se presentan en niveles excesivos en el alimento (Rimblas, 2004).

Tabla 6. Tipos de peligros químicos y algunos ejemplos.

I	**Constituyentes tóxicos naturales** Alimentos marinos: Toxinas de moluscos. Alimentos vegetales: Alcaloides vegetales. Toxinas de hongos (setas): Falotoxina.
I I	**Contaminantes incorporados durante la producción o el almacenamiento** Contaminantes ambientales: Compuestos orgánicos

	persistentes, Policlorobifenilos, Dioxinas, Metales. Contaminantes agrícolas: Plaguicidas, Fertilizantes con nitrógeno. Contaminación por micotoxinas: Aflatoxinas, Ocratoxinas, Tricotecenos, Zearalenona.
III	**Aditivos alimentarios** Conservantes: Nitritos, sulfito sódico, benzoato potásico. Colorantes: Dióxido de titanio, tartracina. Antioxidantes: Ascorbato cálcico, galato de propilo. Edulcorantes: Sorbitol, sacarosa. Emulgentes, estabilizadores, espesantes y gelificantes: Carragenano, metilcelulosa. Otros: Glutamato monosódico.
IV	**Contaminantes generados por tratamientos en la industria alimentaria** Compuestos polares por termooxidación de la fracción lipídica. Formación de compuestos piroorgánicos. Compuestos derivados de aminoácidos y azúcares. Compuestos formados por tratamiento alcalino. Uso de detergentes y desinfectantes. Migraciones de los componentes de los envases.

4.1. Legislación sobre peligros químicos en servicios de restauración

Respecto a la elaboración y comercio de comidas preparadas, la formación de sustancias tóxicas durante los procesos de fritura con aceites y grasas es el único aspecto referente a peligros químicos reflejado en la legislación Española. Sin embargo, a nivel europeo no existe una legislación común por lo que los diferentes países europeos (Tabla 7) establecen sus propios límites o recomendaciones respecto a la degradación de los aceites y grasas de fritura para consumo humano (Dobarganes, 1998; Ollé, 1998).

Tabla 7. Valores europeos máximos permitidos en aceites y grasas de fritura.

PAÍS	COMPUESTOS POLARES	GRADO DE ACIDEZ	PUNTO DE HUMO	DÍMEROS Y POLÍMEROS DE TRIGLICÉRIDOS	ÁCIDOS GRASOS OXIDADOS
AUSTRIA	27 %	2,5	170ºC		1 %
BÉLGICA	25 %	%ácidos grasos libres <2,5	170ºC	10 %	
FRANCIA	25 %				
HUNGRÍA	25 %		180ºC		
ESPAÑA	25 %				
SUIZA	27 %		170ºC		
PORTUGAL	25 %	Los inspectores utilizan el Fritest (Merk) o VeriFry (Libra) si >0 se comprueba el % de ácidos grasos libres			
HOLANDA		4,5		16 %	
ITALIA	25 %				
ALEMANIA	24 %	2	170ºC		0,75 %
LUXEMBURGO	Los inspectores utilizan el Fritest (Merk) si >0 se comprueba el % de ácidos grasos libres, % PC y sabor, color, olor y apariencia				
FINLANDIA	25 %	2,5	170ºC/180ºC		
SUECIA	Los inspectores utilizan el Oxifrit test y %PC como método de referencia				
NORUEGA	Sin referencia específica legislada a nivel nacional. Los inspectores utilizan el Fritest (Merk).				
DINAMARCA					
INGLATERRA					
IRLANDA					

Regulación específica con consecuencias legales si se infringe Recomendaciones sin consecuencias legales

Estos controles son importantes porque durante la fritura se producen cambios y alteraciones en el aceite. Al aumentar la temperatura se aceleran todos los procesos químicos y enzimáticos, favoreciendo su degradación (Billek, 2000). Los principales cambios químicos que se observan en los aceites calentados son:

a) Hidrólisis: el resultado de la hidrólisis es la aparición de ácidos grasos libres, que aumentan la acidez del aceite, y en menor cantidad la formación de metilcetonas y lactonas (Hamilton *et al.,* 1989) que pueden producir aromas desagradables. La aparición de ácidos grasos libres provoca una mayor tendencia a la formación de humo.

b) Autooxidación: la autooxidación es un proceso oxidativo no enzimático, el más frecuente en los procesos de fritura, caracterizado por la oxidación de los ácidos grasos en presencia del oxígeno del aire, dando lugar a compuestos intermedios inestables denominados hidroperóxidos o peróxidos que darán lugar a la formación de radicales libres (Hamilton *et al.,* 1989; Sonntag, 1982). Las grasas que han sufrido un proceso de oxidación tienden a oscurecerse, aumentar la viscosidad, incrementar la formación de espuma y desarrollar gustos y olores anómalos.

c) Polimerización: los radicales libres tienden a combinarse entre ellos o con otros ácidos grasos y forman compuestos lineales, más o menos largos y ramificados, o compuestos cíclicos. Estos polímeros, al ser de mayor tamaño y peso molecular, tienden a aumentar la viscosidad del aceite lo que

por un lado favorece la formación de espuma y, por lo tanto la oxidación, y por otro producen un arrastre mayor de aceite por parte del producto frito debido a que gotea con más dificultad (Choe *et al.*, 2007).

Como resultado de estas tres reacciones de degradación se originan compuestos polares que, si están presentes en elevadas proporciones, además de modificar las características físicas y organolépticas de los aceites, suponen un riesgo para la salud de los consumidores (Johansson *et al.,* 1995). El consumo de aceites y grasas sometidos a sucesivos calentamientos térmicos influye sobre la peroxidación lipídica plasmática y es mayor cuanto mayor sea el número de calentamientos aplicados, por lo que es recomendable no abusar del recalentamiento de los aceites utilizados en la frituras (Abilés *et al.,* 2009). Los efectos derivados de la ingesta de aceites oxidados en animales de experimentación incluyen alteraciones externas (alopecia y piloerección), diarrea, disminución del crecimiento, miopatía, hepatomegalia (puede darse también hipertrofia de los conductos biliares), esteatosis, anemia hemolítica, nefropatía y deficiencias secundarias de vitaminas A y E (Fernández San Juan, 1996; Clarck and Serbia, 1991; Márquez Ruíz and Dobarganes, 1996; López Varela *et al.,* 1995; Flickinger *et al.,* 1997).

Se han descrito efectos graves debidos al consumo de aceites térmicamente oxidados como pérdida de peso y alta mortalidad. Varios autores (Gurr, 1988; Cortesi and Privett*,* 1992) demostraron gran toxicidad en ratas a las que se les administraban dosis intravenosas de hidroperóxidos. Sin embargo, cuando se utilizaron

dosis orales iguales o mayores no se observaron efectos letales. La evidencia indica que los hidroperóxidos no son absorbidos. De hecho la toxicidad de los aceites térmicamente oxidados parece deberse más a compuestos de oxidación secundaria de bajo peso molecular que a los hidroperóxidos como tales (Billek, 2000). Diversos estudios atribuyen a estos productos secundarios los efectos adversos resultantes del consumo de grasas rancias, ya que constituyen sustancias altamente reactivas y tóxicas que pueden modificar proteínas, ácidos nucleicos y otras biomoléculas *in vivo* (Billek, 2000; Cassee *et al.,* 1996; Bombick and Doolittle, 1995; Esterbauer *et al.,* 1991; Comporti, 1993). El consumo de alguno de estos productos secundarios se ha relacionado con la alta incidencia de cáncer de mama y gástrico en países con elevado consumo de grasas insaturadas (Clarck *et al.,* 1991).

La Orden de 26 de enero de 1989 aprueba la Norma de calidad para los aceites y grasas calentados (B.O.E., 1989) y con ella se exige el control de la calidad de los aceites de fritura. Esta Norma obliga a aquellas personas cuya actividad incluye la utilización y manipulación de aceites y grasas comestibles calentadas para elaborar productos alimenticios a mantener ciertas características higiénico-sanitarias. Los aceites y grasas calentados deberán reunir las siguientes características:

1. Estar exentos de sustancias ajenas a la fritura.
2. El contenido en componentes polares será inferior al 25%.
3. Sus caracteres organolépticos serán tales que no transmitan al alimento frito olor o sabor impropio.

4.2. Métodos analíticos de aceites de fritura

Billek *et al.* (1978) realizó la comparación de 4 métodos diferentes (medición del nivel de éter de petróleo en ácidos grasos oxidados, cromatografía en gel de permeación (GPC), cromatografía líquida (LC) en una columna de gel de sílice con un detector de movimiento de alambre y la separación de los componentes polares y no polares en un aceite usado por medio de cromatografía en columna (CC) en gel de sílice para estudiar la calidad del aceite en restaurantes alemanes. Como resultado se observó una buena correlación entre los resultados obtenidos con GPC, LC, y CC y la cantidad de éter de petróleo aislado de los aceites de fritura usados.

Actualmente, la Legislación Española (B.O.E., 1989) establece que el parámetro a tener en cuenta es el porcentaje de los compuestos polares obtenidos por cromatografía en columna de silicagel. Este método se basa en la separación de las grasas calentadas por cromatografía en columna en compuestos polares y no polares, la elución de los no polares y la posterior determinación de los polares por cálculo de la diferencia entre el peso de la muestra añadida a la columna y la fracción no polar eluída. El método evalúa el grado de deterioro de grasas calentadas. Los compuestos polares incluyen sustancias tales como monoglicéridos, diglicéridos, ácidos grasos libres presentes en grasas no calentadas, así como productos transformados durante el calentamiento de la grasa. Los compuestos no polares son principalmente triglicéridos inalterados.

El método propuesto de detección es aplicable a todos los aceites y grasas, tanto animales como vegetales (Waltking and Wessels, 1981). Al tratarse de una técnica de cierta complejidad, algunos autores han intentado definir parámetros válidos para establecer el punto de descarte, como por ejemplo la relación entre la cantidad de polímeros y la constante dieléctrica (Zhang and Addis, 1990); la relación entre compuestos polares, acidez libre y constante dieléctrica (Smith *et al.,* 1986); la acidez libre por sí sola (Dobarganes *et al.,* 1988), la medición de los compuestos polares (Blumenthal, 1988a) y la combinación del color y la acidez libre (Jacobson, 1991) entre otros.

Algunas empresas han comercializado "kits" rápidos y sencillos de realizar (Firestone*,* 2007) que deben ser cotejados con la prueba oficial descrita en el B.O.E., puesto que en ocasiones hemos advertido diferencias notables en los resultados entre ambas técnicas. Esto puede ser debido a falsos positivos producidos por sustancias, en especial colorantes, provenientes del alimento frito (Blumenthal, 1988b). Algunos de ellos son:

-RAU-test o Oxifrit-Test. Para los compuestos oxidados.

-FRITEST. Para los compuestos carbonilos.

-SPOT-test. Para los ácidos grasos libres.

-TESTO 265. Para la determinación de compuestos polares.

-ACM (*Alkaline Contaminant Materials*). Para compuestos alcalinos como jabones.

5. PELIGROS BIOLÓGICOS EN LOS SERVICIOS DE RESTAURACIÓN

La categoría de peligros biológicos puede ser dividida en tres tipos: bacteriano, viral y parasitario (Fos Claver *et al.,* 2000). Las etapas necesarias para la obtención de los alimentos listos para su consumo deben incluir tres cuidados básicos respecto a los peligros biológicos:

1. Destruir, eliminar o reducir el peligro.
2. Prevenir la contaminación.
3. Inhibir el crecimiento y la producción de tóxicos (toxinas).

Cuando el alimento está libre de componentes biológicos perjudiciales para la salud del consumidor se deben tomar medidas para prevenir la contaminación y, si el peligro no fue eliminado totalmente del alimento, el crecimiento microbiológico y la producción de toxinas deben ser inhibidas mediante el control de la a_w y la acidez, la adición de sal o conservantes, etc. (Sun *et al.*, 2005).

5.1. Origen viral

Los virus pasan de un huésped a otro en forma de partículas inertes. Si la vía de transmisión es fecal-oral los virus no siempre dependen de los alimentos como vehículos de transmisión, no obstante pueden también comportarse como agentes infecciosos de enfermedades transmitidas a través de los alimentos (Fos Claver *et al.,* 2000). En general, las enfermedades virales transmitidas por los alimentos y el agua son mucho menos conocidas que el resto.

Esencialmente, todos los virus transmitidos a través de los alimentos, se propagan con las heces e infectan al ser ingeridos (Cliver, 1990). Así como muchos otros agentes infecciosos que se transmiten entéricamente, la mayoría de las infecciones, probablemente, se contraen a través del contacto de una persona con otra, posiblemente al llevar las manos contaminadas de heces a la boca. Si los vómitos son parte de la enfermedad, se pueden propagar partículas virales a través del mismo. La transmisión indirecta de los agentes entéricos puede ocurrir a través de vectores (moscas), fomites (pañales sucios), pero la vía principal es a través de alimentos y agua. Los más frecuentes son Astrovirus, Calicivirus, Picornavirus, Parvovirus, Reovirus, Rotavirus y Adenovirus (Fos Claver *et al.,* 2000), siendo los virus de la hepatitis A y los virus de la gastroenteritis son los que se transmiten con mayor frecuencia a través de los alimentos. Algunos alimentos son especialmente propensos a transmitir virus, como los moluscos y bivalvos, entre los que destacan almejas, berberechos, mejillones y ostiones. Aunque se han dado casos de enfermedades víricas transmitidas por alimentos en servicios de restauración, (Marks *et al.,* 2000; Ozawa *et al.,* 2007) actualmente no existe, a nivel Europeo o nacional, ninguna normativa para la regulación de la presencia de virus en comidas preparadas. En la Tabla 8 se refleja la incidencia de enfermedades causadas por virus a través de alimentos en países europeos. Durante los años recogidos en el informe de la EFSA (2007) en España no se confirmó ningún caso de intoxicación alimentaria producido por virus.

Tabla 8. Brotes alimentarios causados por virus en la Unión Europea (EFSA, 2007).

	VIRUS NO ESPECIFICADOS	VIRUS DE LA ENCEFALITIS POR GARRAPATAS (TBEV)	ADENOVIRUS	CALCIVIRUS Y NORMOVIRUS	VIRUS DE LA HEPATITIS A	ROTAVIRUS	TOTAL
AUSTRIA	-	-	-	6	-	-	6
BÉLGICA	-	-	-	4	-	-	4
DINAMARCA	-	-	-	18	-	-	18
ESTONIA	-	4	-		-	-	4
FINLANDIA	-		-	13	-	-	13
FRANCIA	37	-	-	34	-	-	71
ALEMANIA	-	-	-	150	15	-	165
GRECIA	-	-	-	1	1	1	3
HUNGRÍA	-	-	-	10	-	-	10
IRLANDA	-	-	-	3	-	-	3
ITALIA	-	-	-	-	10	-	10
LETONIA	12	-	1	30	10	107	160
HOLANDA				11			11
NORUEGA				16			16
POLONIA			9	9	3	13	34
ESLOVAQUIA		2					2
ESLOVENIA				22		6	28
SUECIA				31			31
REINO UNIDO				4			4
TOTAL	**49**	**6**	**10**	**362**	**39**	**127**	**593**

5.2. Origen parasitario

Los parásitos pueden estar presentes en los alimentos y en el agua y pueden ser causa de enfermedades. Varían en tamaño desde organismos unicelulares hasta vermes visibles a simple vista. Sus ciclos de vida también varían. Mientras algunos parásitos utilizan un huésped permanente, otros parásitos pasan por una serie de etapas de desarrollo utilizando un huésped diferente, sea animal o humano. Estos parásitos pueden causar una gran variedad de enfermedades, desde algunas con sintomatología leve hasta enfermedades graves y, en algunos casos la muerte.

Los casos de Enfermedades Parasitarias Transmitidas por Alimentos (EPTA) en países desarrollados se han visto incrementados en los últimos años debido a diversas razones, como la globalización del comercio, el consumo de crustáceos crudos o poco cocidos así como los cambios en hábitos de alimentación que nos han llevado a consumir alimentos sin o con escaso tratamiento térmico. El cambio climático y el calentamiento global también han contribuido, haciendo que especies que se consideraban endémicas de zonas tropicales puedan desplazarse y cambiar sus hábitos. Así como ciertos fenómenos naturales que pueden provocar que especies infestadas migren a otros lugares, con lo cual aumenta la aparición de especies poco comunes en algunos países, por ejemplo *Anisakis*.

Todos los alimentos que forman parte de la pirámide alimenticia pueden ser, potencialmente, vehículo de transmisión de parásitos a la especie humana, desde el agua, las frutas y las verduras, los productos cárnicos y piscícolas, así como sus

derivados, hasta todo tipo de producto almacenado, cuyo proceso de conservación no impida la viabilidad de las formas infestantes para la especie humana (Fuentes, 2007).

En función del origen de su presencia en el alimento, se diferencian tres grandes clases:

- Parásitos contaminantes de los alimentos. *Entamoeba histolytica, Giardia intestinalis, Cryptosporidium* spp. y *Balantidium coli.* Son parásitos no propios de los alimentos y que aparecen en éstos como fruto de una contaminación, fundamentalmente de origen fecal.
- Parásitos deteriorantes de los alimentos. Si bien no afectan al hombre, deterioran la calidad y el producto es rechazado. Dentro de éste grupo encontramos artrópodos y otros tipos de parásitos que descomponen el alimento, alteran el aspecto organoléptico producto y disminuyen la calidad del producto final.
- Parásitos propios de los alimentos. *Taenia solium, Taenia saginata, Toxoplasma gondii*, sarcoquistes de *Sarcosystis* spp. y larvas de *Trichinella* spp., *Paragonimus* spp., *Clonorchis sinensis*, *Diphylobotrium* spp., *Gnathostoma* spp., *Anisakis simplex, Phocanema* y *Contracaecum*. Son parásitos propios de los alimentos y destacan por ser patógenos para la especie humana.

5.2.1. Legislación sobre parásitos en servicios de restauración

La presencia de *Anisakis* en pescados está regulada, en España, por el RD 1420/2006, de 1 de diciembre, sobre prevención

de la parasitosis por *Anisakis* en productos de la pesca suministrados por establecimientos que sirven comida a los consumidores finales o a colectividades.

El *Anisakis* es un nematodo (gusano redondo) parásito con un ciclo de vida complejo. Tiene varias fases de vida y en cada una de ellas necesita un hospedador diferente. El ciclo biológico se cierra cuando estos peces y cefalópodos son ingeridos por los mamíferos y grandes peces, que son los huéspedes definitivos (Figura 1).

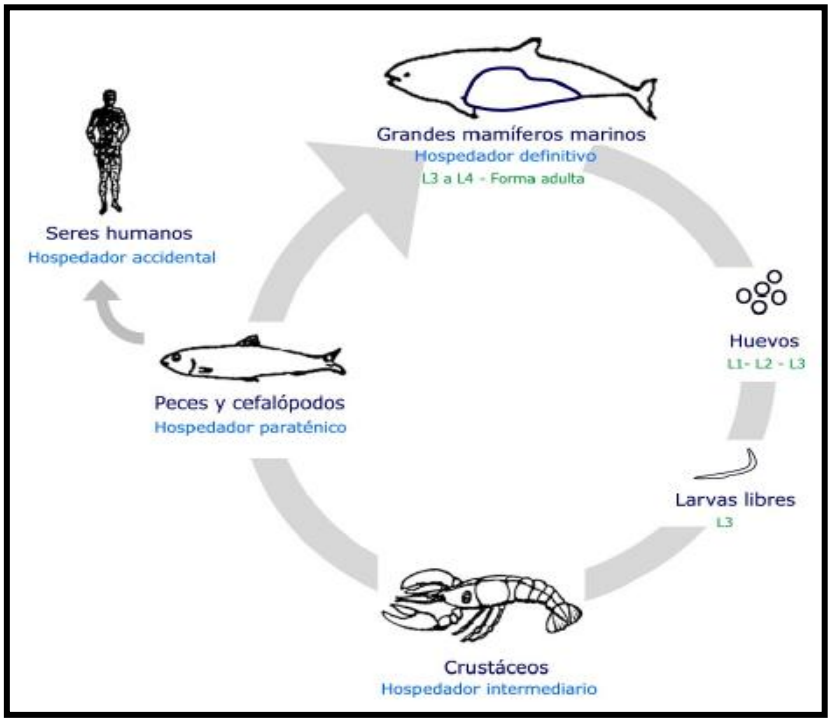

Figura 1. Ciclo biológico del *Anisakis simplex.*

El *Anisakis* se aloja habitualmente en el tubo digestivo de los peces vivos, y una vez que estos mueren, las larvas migran hacia las vísceras y la musculatura, llegando incluso a traspasar la piel

del pescado. Sólo es infectiva para el hombre en la fase de larva 3 (L3), es decir cuando se encuentra en peces, pero no antes (Gago Cabezas *et al.*, 2007).

El hombre es un huésped accidental que puede adquirir las larvas si consume pescado parasitado crudo o poco cocinado. Los primeros casos de parasitación por *Anisakis* se describen en Japón y Holanda (Kagei and Isogaki, 1992), países que presentan un alto consumo de pescado crudo, y posteriormente han ido apareciendo en otros países como España (Cabrera, 2010), Francia (Chord Auger *et al.,* 1995) y Estados Unidos (Shields *et al.,* 2002) entre otros, posiblemente debido a la introducción de nuevas preparaciones culinarias.

Los efectos del *Anisakis* sobre las personas no son por lo general graves. El nematodo anisakis tiene efectos directos e indirectos sobre las personas que se manifiestan con sus respectivas clínicas:

- Efectos directos. Se presentan mayoritariamente con síntomas digestivos (dolor abdominal agudo, nauseas, vómitos y diarreas). Para que se manifiesten estos síntomas se requiere la presencia directa de *Anisakis* vivo.
- Efectos indirectos. Son causados por una reacción alérgica por sensibilización a anisakis que puede provocar desde urticaria a shock anafiláctico, así como efectos sobre la piel, el aparato digestivo o respiratorio.

Las especies parasitadas son diversas, pero entre las más habituales se encuentran el bacalao, sardina, boquerón, arenque,

salmón, abadejo, merluza, pescadilla, caballa, bonito, jurel y calamar.

La cantidad de parásitos varía en función del lugar de captura y del momento de la evisceración. De este modo, los peces capturados en alta mar que son rápidamente eviscerados, presentan menos parásitos que los capturados en la costa.

Las medidas de prevención son básicas para reducir el riesgo de afecciones por *Anisakis* y son sencillas de seguir por parte de los establecimientos que ofrecen pescado a sus clientes. Según el RD 1420/2006 los titulares de los establecimientos que sirven comidas a los consumidores finales o a colectividades (bares, restaurantes, cafeterías, hoteles, hospitales, colegios, residencias, comedores de empresas, empresas de catering y similares) están obligados a garantizar que los productos de la pesca para consumir en crudo o prácticamente en crudo hayan sido previamente congelados a una temperatura igual o inferior a –20 ºC en la totalidad del producto, durante un período de al menos 24 horas; este tratamiento se aplicará al producto en bruto o al producto acabado. También les será aplicable la misma obligación de garantía cuando se trate de productos de la pesca que han sido sometidos a un proceso de ahumado en frío, en el que la temperatura central del producto no ha sobrepasado los 60 ºC, y pertenezcan a las especies siguientes: arenque, caballa, espadín y salmón (salvaje) del Atlántico o del Pacífico. Igualmente estarán obligados a garantizar la congelación en las mismas condiciones si se trata de productos de la pesca en escabeche o salados, cuando este proceso no baste para destruir las larvas de los nematodos. La

Agencia Española de Seguridad Alimentaria y Nutrición (AESAN, 2011) establecerá y difundirá los criterios técnicos necesarios para determinar en estos casos si es necesaria o no la congelación.

5.2.2. Métodos de análisis de anisakidos en pescados

Los peces pueden ser examinados para detectar la presencia de parásitos mediante gran variedad de métodos (EFSA, 2010).

a) Inspección visual

La inspección visual de los filetes revelará gusanos cerca de la superficie, sin embargo, los situados en el interior de la carne no son inmediatamente evidentes. Es el método más sencillo para detectar las larvas del anisakis. Según algunos estudios, mediante este método solo se consiguen detectar el 45-83% de las larvas ubicadas en el músculo de algunos tipos de pescado.

b) Transiluminación

Se utiliza ampliamente para detectar parásitos en la musculatura de los peces. Consiste en proyectar una fuente luminosa por la parte inferior del pescado, normalmente con ayuda de mesas iluminadas. Desafortunadamente, en un estudio reciente se considera que esta técnica tiene baja eficacia ya que con ella solamente se detecta de un 7 a un 10% de las larvas presentes en filetes de pescado infestados (Sivertsen *et al.,* 2011).

c) Digestión

El método de digestión se usa para la detección de parásitos libres en músculo y otros tejidos (Solas *et al.,* 2009). Con éste método se consiguen recuperar prácticamente todos los nematodos anisákidos. Esta técnica consiste en reproducir las condiciones físico-químicas del estómago de los mamíferos para recuperar la mayoría de las larvas existentes en el pescado. Tras aislar las larvas encontradas se debe comprobar su viabilidad. Es una técnica muy eficaz, que permite distinguir entre parásitos vivos y muertos.

d) Métodos moleculares

Recientemente se han desarrollado nuevos métodos caracterizados por su alta especificidad para los géneros *Anisakis, Pseudoterranova, Contracaecum* e *Hysterothylacium* y por una sensibilidad muy elevada, ya que permiten la detección específica del parásito aunque se encuentre en cantidades muy bajas (0.05 pg).

Los métodos moleculares se basan en la técnica de la Reacción en cadena de la Polimerasa (*Polimerase Chain Reaction*, PCR) que permite la detección de los parásitos en el producto analizado a partir de la amplificación y detección de un fragmento específico de su genoma. Este método es rápido, eficaz y puede ser aplicado a cualquier producto pesquero independientemente del grado de transformación que haya sufrido, desde el ejemplar entero, fresco o congelado, hasta conservas y 'surimi', el pescado procesado en barritas (Espiñeira *et al.,* 2010).

5.3. Origen bacteriano

Los migroorganismos de origen bacteriano que pueden suponer un peligro biológico pueden dividirse en tres grupos, de acuerdo a la legislación española (Anonymous, 2001):

a) Organismos indicadores. Su recuento es útil para la evaluación de la inocuidad microbiológica de los alimentos. El análisis microbiológico de alimentos para la búsqueda de estos microorganismos nos permite evaluar:

- Calidad de la materia prima, problemas de almacenamiento, abuso de temperatura, vida útil (Recuento de aerobios mesófilos).
- Potencial contaminación fecal o posible presencia de patógenos (Coliformes fecales).
- Contaminación post tratamiento térmico (coliformes, enterobacterias).

b) Testigos de falta de higiene. Su presencia indica contaminación por manipulación humana (*Staphylococcus aureus* coagulasa positiva) o una potencial contaminación fecal (*Escherichia coli*).

c) Organismos patógenos. Son aquellos que pueden convertir al alimento en el que se encuentran en un potencial vehículo de enfermedad para quien lo consuma. Entre ellos podemos destacar *Salmonella* spp. o *Listeria monocytogenes* entre otros.

En general, el crecimiento bacteriano en alimentos depende tanto de las características del alimento, el agua libre, pH,

potencial de oxidación-reducción, cantidad de nutrientes, etc., como de los tratamientos a los que ha sido sometido y de las condiciones de conservación. Aunque si se dan determinadas condiciones, como pueden ser una mala conservación, escasas condiciones higiénicas, etc., se puede contribuir a favorecer el desarrollo de microorganismos patógenos, que dan lugar a toxiinfecciones alimentarias (Bello *et al.,* 2000; Pszczola *et al.,* 2000). Los alimentos pueden contener microorganismos causantes de intoxicaciones o toxiinfecciones alimentarias. Las intoxicaciones alimentarias de carácter biológico se originan al consumir alimentos que contienen toxinas previamente formadas por el microorganismo, mientras que las toxiinfecciones se producen tras ingerir alimentos contaminados por microorganismos que, al colonizar y multiplicarse en el interior del consumidor, segregan diferentes toxinas (Repetto, 1997). En la Tabla 9 se aprecian los principales agentes causantes de toxiinfecciones e intoxicaciones alimentarias en la Unión Europea (EFSA, 2006).

Tabla 9. Principales agentes causantes de toxiinfecciones e intoxicaciones alimentarias.

	BROTES (%)	**CASOS**	**MUERTES**
Salmonella	**53,9**	**22705**	**23**
Staphylococcus	**4,1**	**2057**	**2**
E. coli	**0,8**	**750**	**1**
Listeria	**0,2**	**120**	**17**
Desconocido	**16,4**	**9437**	**2**

Según el tipo de gérmenes implicados puede tener diversas consecuencias, desde una leve alteración del producto alimentario, como son la pérdida de características organolépticas o el valor comercial, hasta la producción en el consumidor de intoxicaciones y toxiinfecciones con graves consecuencias para su salud.

5.3.1. Legislación sobre bacterias en servicios de restauración.

Atendiendo al aspecto microbiológico, la Reglamentación española recoge en el RD 3484/2000 de 29 de diciembre las normas de higiene para la elaboración, distribución y comercio de las comidas preparadas, y clasifica los tipos de comidas en cuatro grupos:

- Grupo A: comidas preparadas sin tratamiento térmico y comidas preparadas con tratamiento térmico, que lleven ingredientes no sometidos a tratamiento térmico.
- Grupo B: comidas preparadas con tratamiento térmico.
- Grupo C: comidas preparadas sometidas a esterilización.
- Grupo D: comidas preparadas envasadas, a base de vegetales crudos.

La mayor parte de las muestras analizadas pertenecen a los grupos A y B, cuyos criterios microbiológicos se recogen en la Tabla 10.

En ambos grupos se establece como microorganismos indicadores a los aerobios mesófilos y Enterobacteriaceae lactosa positiva o coliformes y como testigos de falta de higiene a *Escherichia coli* y *Staphylococcus aureus*. Además, el Real Decreto

establece la obligatoriedad del análisis para la detección de los patógenos a *Salmonella* spp. y *Listeria monocytogenes*.

Tabla 10. Criterios microbiológicos para el análisis microbiológico en comidas preparadas.

	GRUPO A		GRUPO B	
INDICADORES				
Recuento total de aerobios mesófilos	n=5	m= 10^5	n=5	m= 10^4
	c=2	M= 10^6	c=2	M= 10^5
Enterobacteriaceas (lactosa positiva)	n=5	m= 10^3	n=5	m= 10
	c=2	M= 10^4	c=2	M= 10^2
TESTIGOS DE FALTA DE HIGIENE				
Escherichia coli	n=5	m= 10	Ausencia / g	
	c=2	M= 10^2		
Staphylococcus aureus	n=5	m= 10	n=5	m= 10
	c=2	M= 10^2	c=1	M= 10^2
PATÓGENOS				
Salmonella	n=5	c=0	n=5	c=0
	Ausencia / 25 g		Ausencia / 25 g	
Listeria monocytogenes	n=5	m= 10	n=5	c=0
	c=2	M= 10^2	Ausencia / 25 g	

5.3.1.1. Aerobios mesófilos

En el recuento de aerobios mesófilos se estima la flora total, pero sin especificar tipos de gérmenes. Esta determinación refleja la calidad sanitaria de los productos analizados indicando, además de las condiciones higiénicas de la materia prima, la forma como

fueron manipulados durante su elaboración. Sin embargo, tiene un valor limitado como indicador de la presencia de patógenos o sus toxinas. Un recuento total de aerobios mesófilos bajo no asegura que un alimento esté exento de patógenos o sus toxinas. Un recuento elevado tampoco indica, forzosamente, presencia de flora patógena. Su presencia puede indicar materia prima contaminada, deficientes métodos de manipulación durante la elaboración de los productos y la posibilidad de que entre ellos pueda haber patógenos, dado que esta flora suele ser mesófila. Altos recuentos suelen ser signos de inmediata alteración del producto. Tasas superiores a 10^6-10^7 unidades formadoras de colonias (UFC)/g suelen ser ya inicio de descomposición. En general, el recuento de la flora de aerobios mesófilos es una prueba para conocer las condiciones de salubridad de algunos alimentos (Pascual Anderson, 1999).

5.3.1.2. Enterobacterias

Constituyen un grupo de bacterias que se caracterizan por su capacidad para fermentar lactosa con producción de ácido y gas. Son bacilos gran negativos, aerobios y anaerobios facultativos, no esporulados. Se encuentran en el intestino del hombre y de los animales, pero también en otros ambientes: suelo, plantas, cáscara de huevo, etc. Aunque su especificidad como indicadores no es buena, se suelen usar como índice de contaminación fecal por su frecuencia en heces, y por su fácil detección en el laboratorio. En general, niveles altos de Enterobacterias indican manipulación y elaboración deficientes de los alimentos (Mossel *et al.,* 1995).

5.3.1.3. *Escherichia coli*

Son bacilos gram negativos, oxidasa negativos, catalasa y nitratos positivos, que suelen encontrarse en el suelo, agua, heces, estiércol y tracto digestivo de los animales, siendo las fuentes alimentarias más comúnmente implicadas el agua, leche cruda, productos lácteos y carne (Frazier, 1993; Bourgeis *et al.,* 1995; Lindner, 1995, Sheen and Hwang, 2010). La mayoría de las bacterias pertenecientes a la especie *E. coli* forman parte de la microflora normal del intestino del hombre y de los animales de sangre caliente, encontrándose, habitualmente, en sus heces (Gilligan, 1999; Mead and Griffin, 1998; Nauschuetz, 1998; Lodinova-Zadnikova, 1998). Por su especificidad está considerado como un buen indicador de contaminación fecal en alimentos. La mayor parte de las cepas son inocuas, pero existen algunas que son patógenas para el hombre.

E. coli es uno de los microorganismos más frecuentemente implicados en cuadros y brotes diarreicos, cuyas particularidades en las propiedades de virulencia y síndromes intestinales han conducido a la diferenciación de 5 clases de *E. coli* causantes de la enfermedad diarreica. Las diversas cepas que producen diarreas difieren en sus características clínicas, epidemiológicas y patogénicas pero se semejan en que los alimentos contaminados intervienen en la transmisión y se clasifican en diversos grupos (Nataro and Kaper, 1998) como se muestra en la Tabla 11.

Las cepas virulentas de *E. coli* pueden causar gastroenteritis, infecciones del tracto urinario y meningitis neonatal. La intoxicación alimentaria causada por *E. coli* generalmente es

causada por comer verduras sin lavar o carne poco cocida. La gravedad de la enfermedad varía considerablemente, pues aunque generalmente tiene consecuencias leves, puede ser fatal, especialmente en niños pequeños, ancianos o personas inmunocomprometidas.

Tabla 11. Clasificación y características de los principales tipos de *E. coli*.

MICROORGANISMO	SÍNTOMAS CLÍNICOS	LUGAR DE ACCIÓN	FACTORES DE PATOGENICIDAD
***E. coli* enterotoxigénico (ECET)**	Diarrea acuosa. Diarrea del viajero. Diarrea infantil en países subdesarrollados	Intestino delgado	ST y LT, CFAs, Longus
***E. coli* enteroinvasivo (EIEC)**	Diarrea con moco y sangre o diarrea acuosa seguida de un cuadro disentérico	Intestino grueso	Invasividad, plásmido de 140 Mda
***E. coli* enteropatogénico (EPEC)**	Diarrea infantil con fiebre, náusea, vómitos, heces sin sangre	Intestino delgado	Intimina (A/E), BFP, plásmido EAF
***E. coli* enterohemorrágico (EHEC)**	Colitis hemorrágica, heces con sangre, síndrome urémico hemolítico	Intestino grueso	Stx, intimina (A/E), pO157, toxina Shiga
***E. coli* enteroagregativo (EAEC)**	Diarrea infantil persistente, fiebre ligera	Intestino delgado	Citotoxina, fimbria AAFI, EASTI, proteínas Pet y Pic
***E. coli* enteroadherente (DAEC)**	Diarrea acuosa sin sangre	Intestino delgado	Fimbria F1845, OMP

Como la mayoría de los patógenos de la mucosa intestinal, *E. coli* sigue una estrategia de infección por adhesión, colonización del sitio mucoso, evasión de las defensas del hospedador,

multiplicación y daño en el organismo hospedador. La habilidad más característica de las cepas diarreicas de *E. coli* es la de colonizar la superficie de la mucosa intestinal a pesar de la peristalsis y la competencia por nutrientes de la flora autóctona del intestino (Nataro and Kaper, 1998).

a) E. coli enterotoxigénico (ECET)

*E*s el patógeno más importante de diarrea en bebés, niños y adultos, lo que representa a 280 millones de casos y muertes de más de 400.000 al año (WHO, 2005). ETEC es endémica en muchos países en desarrollo, y sus síntomas se manifiestan con frecuencia en turistas, miembros de las fuerzas armadas u otros visitantes (Coster *et al.,* 2007) ETEC es el patógeno más común de la diarrea del viajero que afecta a 10 millones de visitantes en los países en vías de desarrollo (Aranda-Michel and Gianella, 1999). Además de la diarrea del viajero, ETEC puede causar síntomas de enfermedad clínicamente indistinguible de la enfermedad de cólera causada por *Vibrio cholerae* (Vicente *et al.,* 2005). Entre 10 y 72 horas después de la ingestión de alimentos o agua contaminada, se produce un cuadro de diarreas líquidas acuosas, dolor abdominal, vómitos y a veces fiebre baja, pudiendo llegar a la deshidratación.

Se transmite por la ingestión de alimentos y en algunos casos, de agua contaminada. Las contaminaciones se producen debido a malas prácticas en la manipulación de los alimentos, deficiente conservación de los productos elaborados, y por formas descuidadas en las ofertas de productos alimenticios.

b) Enterotoxinas producidas por ECET

ECET produce una toxina termoestable (*stable toxin; ST*) y una termolábil (*labile toxin; LT*), semejándose esta última a la colérica. La toxina ST es pequeña, monomérica y contiene múltiples residuos de cisteína, cuyas uniones son las responsables de la estabilidad al calor de estas toxinas. ST se considera una causa importante de diarrea en cerdos pero rara vez se asocia con los seres humanos (Guerrant *et al.,* 1990; Lortie *et al.,* 1991; Handl *et al.,* 1992).

La toxina termolábil es el principal factor de virulencia de ETEC (Holmgren and Svennerholm, 1992). Las graves pérdidas de agua y electrolitos que se producen en la infección por *E. coli* parecen ser causados por esta toxina cuya acción está mediada por la estimulación de la actividad de la adenilato ciclasa en las células epiteliales del intestino delgado (Kantor *et al.,* 1974). En este sentido, la enterotoxina LT de *E. coli* muestra algunas características similares a la enterotoxina producida por *Vibrio cholera* (*cholera toxin; CT*) (Sharp *et al.,* 1973). Tanto en términos de estructura como de función ambas toxinas, están estrechamente relacionadas y son también muy similares. La LT y la CT son representativas de un tipo de toxinas heteromultiméricas AB producidas por una serie de patógenos bacterianos (Williams *et al.*, 1999). A pesar de que LT B y CT B sólo presentan una diferencia del 20% en sus secuencias de nucleótidos y aminoácidos, se distinguen por sus propiedades individuales bioquímicas e inmunológicas (Hol *et al.,* 1995; Merrit *et al.,* 1996). Las dos están formadas por un pentámero con cinco subunidades

B idénticas y una subunidad A catalítica (Mudrak and Kuenh, 2010). LT A y LT B se componen de 236 y 103 aminoácidos y tienen masas moleculares de 27-28 kDa y 11,9 kDa respectivamente (Mekalanos *et al.,* 1983). La subunidad A es enzimáticamente activa y es la responsable de la toxicidad, mientras que la subunidad B que se une al receptor, y facilita la entrada del dominio A en las células eucariotas. El tratamiento térmico de la toxina LT rompe el anillo pentamérico en monómeros, liberando la subunidad A. Aunque la actividad catalítica está presente en LT A libre, el pentámero LT B se requiere para la entrada en las células del epitelio intestinal, y por lo tanto, la rotura de la holotoxina por tratamiento térmico previene la intoxicación de las células huésped (Mudrak and Kuenh, 2010).

5.3.1.4. *Staphylococcus aureus*

S. aureus es un coco Gram positivo, anaerobio facultativo y catalasa y coagulasa positivo. Es el agente etiológico de diversas patologías, incluyendo infecciones de piel y tejidos blandos, bacteremia, endocarditis, infección de sistema nervioso central y del tracto genitourinario (Waldvogel, 2009). Al ser un microorganismo que se encuentra de forma habitual en la piel, fosas nasales y mucosas del ser humano, su presencia en los alimentos refleja unas prácticas de manipulación defectuosas y es signo evidente de falta de higiene. *S. aureus* es un microorganismo muy resistente, a las condiciones ambientales, y extremadamente difícil de erradicar. Pese a que no es esporulado, soporta bien condiciones extremas aunque se inactiva a

temperatura de congelación y puede eliminarse con una cocción correcta (Smith *et al.,* 2001). Las principales fuentes alimentarias implicadas en las intoxicaciones por *S. aureus* son la leche y los productos lácteos, carnes y productos cárnicos, pescado ahumado, ensaladas y ovoproductos.

En ocasiones puede no detectarse *S. aureus* en un alimento o estar presente en bajo número pero, sin embargo, puede existir cantidad detectable suficiente de enterotoxina estafilocócica. En este caso, los gérmenes que originaron la toxina han ido descendiendo en número e, incluso, desaparecen, mientras que la toxina, por su mayor resistencia, permanece en el alimento dando curso a la enfermedad.

Ésta bacteria produce una gran variedad de exoproteínas que contribuyen a su habilidad para colonizar el intestino y causar daño en el hospedador. Algunas cepas de *Staphylococcus* producen una o más exoproteínas adicionales, entre ellas se incluyen la Toxina del Síndrome del Shock Tóxico (TSST) y las enterotoxinas estafilocócicas (SEs). Las SEs son sintetizadas por especies del género *Staphylococcus*, mayoritariamente *S. aureus*. Son proteínas cuyo peso molecular oscila entre 26 y 30 KDa y están compuestas por una cadena simple de aminoácidos plegada en forma globular (Balaban and Rasooly, 2000). Actualmente se conocen 23 tipos diferentes de enterotoxinas, nombradas de SEA a SEV (Schlievert and Case, 2007). La originalmente descrita como enterotoxina F fue más tarde identificada como la toxina productora del síndrome de shock tóxico (TSST-1) en mujeres y en ocasiones en hombres. TSST actúa como superantígeno llevando a

una liberación masiva de citoquinas incluyendo el factor alfa de necrosis tumoral (TNF-alfa), la interleucina-1 (IL-1) e IL-6, que son responsables del desarrollo de los signos clínicos típicos. El Síndrome del Shock Tóxico (SST) producido por la toxina TSST consiste en una enfermedad aguda multisistémica caracterizada por la aparición repentina de fiebre, vómitos y diarreas, hipotensión, enrojecimiento conjuntival y exantema (con posterior descamación). Investigaciones actuales indican que la producción de TSST-1 por cepas de *S. aureus*, mantiene una relación directa con algunos serotipos toxigénicos que provocan brotes de intoxicación alimentaria. Estos estudios han sido realizados en cepas aisladas en humanos, alimentos y animales domésticos (Adesiyun *et al.,* 1992). Sin embargo, las aisladas en alimentos son mayoritariamente las enterotoxinas A, B, C, D. SEA concretamente es la enterotoxina más frecuentemente aislada en los brotes de intoxicaciones alimentarias, seguida por SED (37, 5%) y SEB (10%) (Balaban and Rasooly, 2000). Cuando se ingiere un alimento con enterotoxina estafilocócica y después de un período de incubación de 1-8 horas se produce un síndrome gastrointestinal, que se caracteriza por la aparición de náuseas, cefaleas, dolores abdominales y sobre todo vómitos violentos y repetidos, a menudo acompañados de diarrea (Dinges *et al.,* 2000; Wilkinson *et al.,* 1997; Marrack *et al.,* 1990).

5.3.1.5. *Listeria monocytogenes*

Son bacilos Gram positivos, cortos, con extremos redondeados y, a veces, puntiagudos (cocobacilos). Su hábitat es el intestino del

hombre sano, mamíferos domésticos y salvajes. También se encuentra en el suelo, aguas residuales, fango, plantas, vegetales marchitos, forraje, abonos naturales, desagües de mataderos y agua de rio principalmente. Las fuentes alimentarias más frecuentemente implicadas en intoxicaciones por *L. monocytogenes* son quesos, leche, carne, frutas y verduras (Frazier *et al.,* 1993; Bourgeis *et al.,* 1995; Lindner, 1995; Pascual Anderson, 1989). El período de incubación es muy variable (de 8 días a 3 meses) presentando un mecanismo de patogenicidad doble. Por un lado posee una elevada capacidad invasiva y por otro lado es capaz de sintetizar una toxina con propiedades altamente hemolíticas (hemolisina). *L. monocytogenes* es el patógeno causante de listeriosis en el ser humano con tres manifestaciones primarias: meningitis, aborto y septicemia perinatal. La meningitis afecta a personas de alrededor de 60 años de edad, generalmente inmunosuprimidas y su pronóstico es variable. Sin embargo, en mujeres embarazadas, puede dar lugar a la interrupción del embarazo o al nacimiento de niños con meningitis o septicemias. El contagio suele producirse por vía transplacentaria o transgenital durante el parto, siendo el aborto el síntoma más frecuente entre el 5º y 6º mes de gestación. La listeriosis septicémica suele ser benigna cuando afecta a personas sanas, cursando con síntomas parecidos a los de la gripe como fiebre, malestar y alteraciones intestinales. Esta toxiinfección ha experimentado un incremento alarmante en los últimos años, lo que unido a su carácter ubicuo hace especialmente necesaria su vigilancia y control (Taege *et al.,* 1999).

5.3.1.6. *Salmonella* spp.

Salmonella corresponde a un género bacteriano perteneciente a la familia Enterobacteriaceae integrado por gérmenes de forma bacilar, habitualmente móviles. Son bacterias gran negativas, anaerobios facultativos, que fermentan glucosa con producción de gas, no fermentan la lactosa y reducen los nitratos. Se encuentran ampliamente distribuidos en la naturaleza a través de materias fecales, agua, suelo, etc. Su reservorio primario es el tracto intestinal de vertebrados de sangre caliente y fría, así como insectos. *Salmonella* es una de las enterobacterias que causa importantes afecciones en la salud del hombre, puede contraerla cualquier persona aunque es más común que la padezcan lactantes, niños y ancianos. Se trata de una enfermedad que afecta habitualmente a la zona intestinal y en ocasiones a la circulación sanguínea. Los síntomas aparecen generalmente de uno a tres días después de la exposición, con diarreas, fiebre, dolor abdominal, cefalea y ocasionalmente vómitos, también pueden ocasionar septicemias, osteomielitis y meningitis. Las bacterias presentes en los alimentos se multiplican hasta cantidades millonarias cuando éstos son expuestos a malas condiciones de conservación, a temperatura ambiente y por tiempo prolongado entre la elaboración y el consumo. La bacteria puede ser transmitida a través del alimento contaminado, al beber agua contaminada o por utensilios, superficies de trabajo o mesas previamente usadas para preparar alimentos que se encontraban contaminados. Otra manera de contraer la enfermedad es teniendo contacto directo con heces fecales de una persona o animal infectado y después

transferir las bacterias de las manos a la boca. Estas contaminaciones son más frecuentes por la participación de moscas y otros vectores. Las frutas y vegetales mal lavados pueden estar contaminados por el abono proveniente de las heces de animales (Warlow *et al.,* 1994; Clearly *et al.,* 1997; Valdés-Dapena Vivanco *et al.,* 2001). Los estudios de los brotes de *Salmonella* han permitido conocer los alimentos más relacionados con esta enfermedad entre los que están las carnes sin procesar, huevos, productos sin pasteurizar de la leche y el queso, productos caseros que contienen huevos crudos, como mayonesas, verduras, coco, y otros productos (Frazier *et al.,* 1993; Bourgeis *et al.,* 1995; Lindner, 1995).

5.3.2. Métodos de análisis de bacterias y toxinas bacterianas

5.3.2.1. Medios de cultivo

Para que las bacterias crezcan adecuadamente en un medio de cultivo artificial éste debe reunir una serie de condiciones como son: temperatura, grado de humedad y presión de oxígeno adecuadas, así como un grado correcto de acidez o alcalinidad. En un medio de cultivo adecuado deben estar presentes los nutrientes y factores de crecimiento necesarios y debe estar exento de todo microorganismo contaminante.

Existen una gran variedad de medios diferentes para el crecimiento de los diferentes tipos de microorganismos (Allaert and Escola, 2002), atendiendo a su utilidad práctica podemos clasificarlos en:

a) Medios de preenriquecimiento: Son aquellos que poseen los componentes mínimos para que pueda producirse el crecimiento de bacterias que no necesiten requerimientos especiales. Sus nutrientes permiten el crecimiento de gran número de microorganismos. Algunos de los medios más conocidos de este grupo son el agua de peptona tamponada o el caldo tripticasa de soja.

b) Medios selectivos: Son medios utilizados para favorecer el crecimiento de ciertas bacterias contenidas en una población polimicrobiana. El fundamento de estos medios consiste en facilitar nutricionalmente el crecimiento de una población microbiana específica, al mismo tiempo que se inhibe el crecimiento de otros microorganismos que interferirían con el que estamos buscando. Un ejemplo de medio selectivo es el caldo selenito, que se utiliza para favorecer el crecimiento de *Salmonella* y frenar el del resto de enterobacterias.

c) Medios de cultivo diferenciales: Utilizan propiedades diferenciales del crecimiento microbiano. La adición de un azúcar fermentable o un sustrato metabolizable por un tipo específico de bacterias se utiliza para diferenciar al microorganismo de interés. El medio MacConkey es un medio diferencial porque permite distinguir los gérmenes que fermentan la lactosa de aquellos que no lo hacen, el medio de cultivo con agar sangre diferencia las bacterias hemolíticas de las no hemolíticas.

5.3.2.2. Sistemas de identificación bacteriana

Se entiende por identificación bacteriana al conjunto de técnicas y procedimientos que se aplican para establecer la identidad de un microorganismo. Para realizar una identificación presuntiva rápida o una confirmativa, se suelen utilizar numerosas pruebas. Entre ellas se incluyen la observación de la morfología colonial, las reacciones de tinción o los requerimientos del cultivo. En ocasiones la información obtenida con los ensayos anteriores no es suficiente y debemos recurrir a otro tipo de pruebas que nos aporten información más concreta para la identificación bacteriana (Allaert and Escola, 2002).

5.3.2.2.1. Pruebas bioquímicas

Generalmente, los microorganismos se cultivan en medios que contienen una sustancia nutritiva específica y después de la incubación, el cultivo se examina para ver los cambios químicos que hayan ocurrido. El tiempo necesario para la identificación de bacterias puede reducirse considerablemente con el uso de sistemas miniaturizados basados en pruebas bioquímicas. Muchos de estos sistemas permiten la realización de varias pruebas bioquímicas simultáneamente. Cada uno de los ensayos, consta de tubos miniaturizados que contienen el medio de cultivo que se hidrata al inocularlo con la suspensión bacteriana pura. Las pruebas se clasifican en grupos; a cada uno de resultados positivos de los ensayos de se le asigna un determinado valor numérico, obteniéndose un código que corresponderá a un determinado género o especie en un texto de la base de datos.

Algunos ejemplos de este tipo de sistemas son Rapid ONE System o las tiras API. Una limitación de este tipo de método de identificación es la aparición de cepas mutantes y la adquisición de plasmidios que pueden dar origen a cepas con características diferentes (Allaert and Escola, 2002).

5.3.2.2.2. Tipificación con fagos

La interacción entre un virus bacteriano (fago) y su célula bacteriana sensible es sumamente específica, ya que el proceso de adsorción se encuentra mediado por receptores específicos tanto en el virus como en la célula bacteriana. El uso de fagos específicos permite identificar y subclasificar bacterias dentro de una misma especie.

5.3.2.2.3. Pruebas moleculares

En el uso de métodos basados en biología molecular permite, a través de procedimientos y reactivos, detectar determinadas secuencias de ADN que son propias de un determinado agente microbiano.

El método más ampliamente utilizado es la PCR, que se aplica generalmente para la identificación de microorganismos que no pueden ser cultivados por los métodos convencionales (Berrada *et al.,* 2006a). A través de este método, puede aumentarse la cantidad de ADN hasta niveles detectables mediante electroforesis o mediante sondas de ADN.

5.3.2.2.4. Técnicas inmunológicas

La detección de antígenos nos permite identificar la presencia de microorganismos o de fragmentos de los mismos en las muestras.

Los métodos serológicos, implican la utilización de preparaciones de inmunoglobulinas específicas provenientes del suero o de un reactivo. Cada uno de los métodos tiene su fundamento particular, pero en líneas generales, todos se basan en la reacción de un antígeno presente en el agente microbiano con su anticuerpo correspondiente. Inmunoensayos del tipo enzimoinmunoensayos (*Enzyme Linked ImmunoSorbent Assay*, ELISA) han sido desarrollados para la detección e identificación de varios tipos de agentes microbianos (Kuo *et al.,* 2010).

5.3.2.3. Métodos de detección de toxinas bacterianas

Existe gran variedad de métodos para la detección de toxinas bacterianas y, dependiendo de las características de la toxina analizada pueden seleccionarse diversas técnicas.

Las técnicas basadas en reacciones inmunológicas han sido ampliamente utilizadas, existen métodos muy diversos como son la inmunocromatografía, los enzimoinmunoensayos del tipo ELISA, la inmunofluorescencia, etc. Incluso la combinación de sistemas inmunológicos con moleculares (PCR-ELISA, Inmuno-PCR), o únicamente técnicas moleculares como los métodos genotípicos basados en hibridación de ácidos nucleicos (GenQuence, sistema VIT, Accuprobe, etc.) y microarrays (FoodExpert) y basados en la amplificación de ácidos nucleicos: PCR (Bactotype, etc.), PCR en tiempo real (sistema A-BAX, iQ-

Check, TaqMan detection kits, LightCycler kits, etc.), NASBA (NucliSens EasyQ Enterovirus) y genotipado de cepas (Riboprinter) ofrecen otras posibilidades en la detección de toxinas (Berrada *et al.,* 2006b).

Las técnicas de aglutinación han sido unas de las más empleadas para la identificación de toxinas bacterianas hasta el momento. La hemoaglutinación con látex en fase reversa (*Reverse Phase Latex Agluttination*, RPLA) se fundamenta en que partículas de este producto sensibilizadas con antienterotoxinas purificadas pueden aglutinar con la presencia de la enterotoxina homóloga; al entrar en contacto los anticuerpos mediante los dos puntos de unión con el antígeno establecen entramados de muchas moléculas de antígeno y de anticuerpos. El kit RPLA es una prueba de aglutinación pasiva comúnmente usada para la determinación de toxinas de *S. aureus*, *C. perfringens*, *E. coli*, *B. cereus* y *C. botulimun* (Balaban and Rassoly 2000; Toma *et al.,* 1999; Beutin *et al.,* 1996; Rusul and Yaacob, 1995).

La aplicación de técnicas cromatográficas acopladas a diversos sistemas de detección de espectrometría de masas ofrece muchas posibilidades para el desarrollo de nuevas técnicas analíticas con el fin de detectar toxinas procedentes de microorganismos contaminantes de alimentos.

a) Toxina termolábil de *E. coli*

La detección de la LT de *E. coli* se ha llevado a cabo por varios métodos, incluyendo desde técnicas inmunológicas muy diversas (Evans and Evans, 1977; Honda *et al.,* 1981a) a técnicas de detección utilizando cultivos de líneas celulares in vitro (Spiers *et*

al., 1977). Una de las técnicas aplicadas con mayor frecuencia son los ensayos de hibridación de ADN (Galbadage *et al.,* 2009) y las pruebas de aglutinación en látex (Gulhan *et al.,* 2009). Sin embargo, la cuantificación de la producción de LT se ha realizado principalmente con los métodos basados en la captura de enzimas, como el ensayo inmunoabsorbente GM1-ligado a enzimas (ELISA-GM1) (Hedge *et al.,* 2009) y la prueba ELISA de anticuerpos de captura (cELISA) (Lasaro *et al.,* 2007). Estas técnicas son ampliamente aceptadas, ya que son sensibles, específicas, rápidas y fáciles de realizar.

b) Toxinas estafilocócicas

Actualmente existen una gran variedad de técnicas disponibles para la detección de toxinas estafilocócicas en diferentes matrices. Las primeras investigaciones para la obtención y purificación de toxinas estafilocócicas comenzaron a principios de la década de los 60 y se purificaron diferentes serotipos toxigénicos a partir de cepas productoras de enterotoxinas mediante varias combinaciones de cromatografía de intercambio iónico y filtración sobre gel. Las primeras purificaciones empleaban mucho tiempo y los resultados no siempre eran los esperados. Más tarde, en diversos estudios realizados para la producción y purificación de las enterotoxinas de los serotipos B y E se obtuvieron mejores resultados y un alto grado de pureza (Bécquer *et al.,* 1989; 1992). En las Tablas 12, y 13 se muestra una visión general de los métodos recientes más utilizados y sus límites de detección.

La purificación de las toxinas y la demostración de su antigenicidad ha permitido emplear métodos serológicos para la

detección de éstas en filtrados de cultivos. Los métodos serológicos utilizados inicialmente fueron la difusión simple (tubo de Oudin); la inmunodifusión radial simple, la inmunodifusión radial doble (Ouchterlony), la placa de óptima sensibilidad (OSP), y el método de la lámina.

Para el estudio de brotes de intoxicación alimentaria y la investigación de la procedencia humana de las cepas toxigénicas aisladas se han realizado también pruebas de fagotipificación y técnicas basadas en el ADN recombinante (Hécquer *et al.,* 1991; Lapinet *et al.,* 1996; Wyat*,* 1995; Betts*,* 1995; Matsusaky*,* 1995). La electroinmunodifusión y la hemoaglutinación, también han sido utilizadas, pero se han observado reacciones no específicas con algunos de los componentes de diversos alimentos (Ng and Tay, 1993).

El radioinmunoensayo (RIA) puede detectar niveles bajos de toxinas en un tiempo corto pero requiere de equipos costosos y condiciones especiales de trabajo. La detección de enterotoxinas por el método inmunoenzimático (ELISA), que detecta iguales concentraciones con similar consumo de tiempo que el RIA, es el más factible en muchos laboratorios por la multiplicidad de determinaciones que pueden realizarse, aunque en ocasiones presenta algunos inconvenientes en lo referente a los componentes de ciertas matrices alimentarias, que afectan a la selectividad y la sensibilidad del proceso. La prueba RPLA es muy sensible pues detecta cantidades entre 1 y 2 ng/mL y requiere un tiempo de 24 h para su ejecución (Andrews and Messer, 1990). La disponibilidad en el mercado de este sistema nos permite

utilizarlo tanto para la detección de toxinas estaphylocócicas (SEs, TSST) como de otros tipos de toxinas bacterianas.

En general, la utilización de técnicas inmunológicas supone métodos simples y de elevada sensibilidad, aunque presentan también algunas desventajas. Muchos de ellos no son capaces de distinguir entre enterotoxinas activas e inactivas; en ocasiones se producen reacciones cruzadas con antígenos no relacionados, originando con ello falsos positivos o falsos negativos. Algunas de estas técnicas requieren también elevados periodos de incubación, lo que retrasa el tiempo de análisis.

En los últimos años la utilización de técnicas cromatográficas así como de la espectrometría de masas está adquiriendo gran importancia en el campo de la proteómica aplicada a ciencias de la alimentación (Carbonaro, 2004). Diversos autores han aplicado técnicas de separación electroforética y detección por espectrometría de masas mediante desorción/ionización láser asistida por matriz acoplado a un detector de iones por tiempo de vuelo (*matrix-assisted laser desorption/ionization-time of flight mass spectrometry; MALDI-TOF*) para la detección de SEs, así como técnicas de LC/MS o LC/MS-MS con resultados satisfactorios.

Los métodos más utilizados hasta el momento para la detección de este tipo de toxinas en servicios de restauración han sido el SET-RPLA (Soriano *et al.,* 2002d; Udo *et al.,* 1999; Al Bustana *et al.,* 1996; Soriano *et al.,* 2002e), las técnicas moleculares como la PCR, (Berrada *et al.,* 2006a; Udo *et al.* 2009; Landeiro *et al.,* 2007), los métodos serológicos como *slide gel diffusion method* (Moore and Bower, 1971) u otros métodos inmunológicos como son

immunoassay test kit RIDASCREEN SET (Ostyn *et al.,* 2011) o la doble difusión en gel (Sule *et al.,* 2007).

Tabla 12. Utilización del SET-RPLA en diferentes matrices para la detección de enterotoxinas estafilocócicas durante los últimos 10 años.

ENTEROTOXINAS	FUENTE	REFERENCIA
A,B,C,D	Cultivos de *S. aureus* aislados de puré de patatas elaborado con leche cruda de bovino, leche sin tratar y salchichas	Jorgensen *et al.,* 2005a
A,B,C,D	Muestras fecales de pacientes asociados con brotes de origen alimentario	Chen *et al.,* 2001a
A,B,C,D	Muestras aisladas de casos de intoxicación alimentaria	Chiang *et al.*, 2006
A,B,C,D	Alimentos relacionados con casos de intoxicación alimentaria	Kochman *et al.,* 2005
A,B,C,D	Alimentos manipulados manualmente	Fueyo *et al.,* 2005b
A,B,C,D	Carne de pollo cruda	Kitai *et al.*, 2005
A,B,C,D	Queso Domiati fresco y curado (queso suave de Egipto)	El-Sharoud *et al.,* 2008
A,B,C,D	Muestras de alimentos y muestras de superficies de contacto con los alimentos	Normanno *et al.,* 2005
A,B,C,D	Empleados y muestras de alimentos	Martín *et al.,* 2004
A,B,C,D	Leche cruda	Tsegmed *et al.,* 2007

Tabla 12. (Continuación).

ENTEROTOXINAS	FUENTE	REFERENCIA
A,B,C,D	Leche, queso, helado, postres y aperitivos	Aragon-Alegro *et al.,* 2007
A,B,C,D	Muestras de las cavidades nasales de portadores sanos, heridas superficiales, quesos, pasteles y otros alimentos manipulados manualmente.	Fueyo *et al.,* 2001
A,B,C,D	Huevos revueltos	Miwa *et al*., 2001
A,B,C,D	Leche de vaca, cabra y productos lácteos	Loncarevic *et al.,* 2005
A,B,C,D	Productos a base de pescado	Simon *et al.,* 2007
A,B,C,D	Muestras de leche de vacas con mastitis	Wang *et al.,* 2009
A,B,C,D	Cerdo crudo y jamón ahumado	Atanassova *et al*., 2001
A,B,C,D	Productos lácteos	Veras *et al.,* 2008
A,B,C,D	Leche y productos lácteos	Cremonesi *et al.,* 2005
A,B,C,D	Comida vietnamita	Huong *et al.,* 2010
A,B,C,D	Portadores nasales	Fueyo *et al.,* 2005a
A,B,C,D	Alimentos listos para tomar	Su *et al.,* 2007
A,B,C,D	Carcasas de cerdo	Nitzche *et al.,* 2007
A,B,C,D	Muestras de leche de vacas con mastitis	Stephan *et al.,* 2001
A,B,C,D	Leche y productos lácteos	Normanno *et al*., 2007b
A,B,C,D	Leche y productos lácteos	Morandi *et al*., 2007

Tabla 12. (Continuación).

ENTEROTOXINAS	FUENTE	REFERENCIA
A,B,C,D	"Dobles" listos para comer (bara, Channa y salsa)	Mankee *et al.*, 2005
A,B,C,D	Leche desnatada en polvo	Ikeda *et al.,* 2005
A,B,C,D	Leche de vaca, cabra y productos lácteos	Jorgensen *et al.,* 2005b
A,B,C,D	Alimentos sospechosos de producir intoxicaciones estafilocócicas	Kérouanton *et al.,* 2007
A,B,C,D	Productos de panadería, bocadillos, leche, queso blanco, Sandwiches, carne de cerdo	Cunha *et al.,* 2006
A,B,C,D	Leche de vacas con mastitis, carne y verduras crudas	Moon *et al.,* 2007
A,B,C,D	Muestras clínicas y de alimentos	El-Ghodban *et al.*, 2006
A,B,C,D	Muestras de leche de vacas con mastitis	Boynukara *et al.,* 2008
A,B,C,D	Muestras clínicas de recién nacidos hospitalizados	Cunha *et al.,* 2007
A,B,C,D	Muestras de manipuladores de alimentos	Loeto *et al.,* 2007
A,B,C,D	Bolitas de patata	Nema *et al.,* 2007
A,B,C,D	Pollo empanado	Pepe *et al.,* 2006
A,B,C,D	Carne y productos lácteos	Normanno *et al.*, 2007a
A,B,C,D	Pacientes relacionados con enfermedades por intoxicaciones alimentarias	Chiang *et al.*, 2008
A,B,C,D	Vegetales,huevos, pescado, carne y pasta	Soriano *et al.*, 2002d

Tabla 13. Métodos de detección de enterotoxinas estafilocócicas y TSST-1 durante los últimos 10 años.

TOXINA	MÉTODO	FUENTE	REFERENCIA
SEA-SEE	VIDAS SET	Leche y productos lácteos	Morandi *et al.*, 2007
SEA-SEE	VIDAS SET	Leche y productos lácteos	Cremonesi *et al.,* 2005
SEA-SEE	VIDAS SET with a Staph strip	Alimentos listos para tomar	Oh *et al.,* 2007
SEA-SEE	VIDAS SET	Leche	Montenegro Stamford *et al.,* 2006
----	Vidas Staph enterotoxin II (ELFA)	Carcasas de cerdo	Hassler *et al.*, 2008
SEA-SEE	VIDAS SET	Alimentos portugueses	Pereira *et al.,* 2009
SEA-SEE	VIDAS SET	Leche procesada a partir de leche en polvo desnatada	Soejima *et al.,* 2007
SEA	VIDAS SET	Productos lácteos	Soejima *et al.,* 2007
SEA-SEE	TECRA kit	Leche de vacas con mastitis	Boerema *et al.,* 2006
SEA-SEE	TECRA kit	Carne fresca y procesada	Al-Tarazi *et al.,* 2009
SEA-SEE	TECRA kit	Producto tradicional fermentado a base de carne de cerdo	Chokesajjawatee *et al.,*2009
SEA-SEE	TECRA kit	Kimbap listo para tomar	Lee *et al.,* 2007
SEA-SED	TECRA kit	Patrones de enterotoxinas	Portocarrero *et al.*, 2002
SEA-SEE	Ridascreen SET	Sufu (cuajada de soja fermentada)	Han *et al.,* 2001
SEA-SEE	Ridascreen SET	Alimentos	Rosec *et al.,* 2002

Tabla 13. (Continuación).

TOXINA	MÉTODO	FUENTE	REFERENCIA
SEA-SEE	Transia plate-staphylococcal enterotoxin kit	Puré de patatas	Jorgensen *et al.,* 2005a
SEs	Transia plate-staphylococcal enterotoxin kit	Producto fermentado a base de carne de cerdo	Chokesajjawatee *et al.,*2009
SEA-SEE	Transia plate-staphylococcal enterotoxin kit	Queso	Ikeda *et al.,* 2006
SEs	Transia plate-staphylococcal enterotoxin kit	Leche desnatada en polvo	Ikeda *et al.,* 2005
SEA-SEE	Transia plate-staphylococcal enterotoxin kit	Postres japoneses	Shimamura *et al.,*2006
SEH	Sandwich ELISA	Puré de patatas	Jorgensen *et al.,* 2005a
SEA-SEG	ELISA	Comida preparada	Rahimifard *et al.,*2008
SEA- SEE	ELISA	Carne y productos lácteos	Nájera-Sánchez *et al.*, 2003
SEA-SEE	ELISA	Alimentos	Rosec *et al.,* 2002
SEA-SEE	ELISA	Helados	Tasci *et al.,* 2011
A,B,C,D	Western blotting	Bolitas de patata	Nema *et al.,* 2007
SEA-SEH, TSST	Western blotting	Alimentos, cepas puras y pacientes	Zell *et al.*, 2008
SEA-SED	Western blotting	Pollo empanado	Pepe *et al.,* 2006
SEH	Western blotting	Leche desnatada en polvo	Ikeda *et al.,* 2005
SEA-SEE	Ridascreen SET	Queso de cabra	Akineden *et al.,* 2008
SEA-SED	Staphilococcal Enterotoxin Test Kit	Vacas con mastitis	Cenci-Goga *et al.,* 2003

Tabla 13. (Continuación).

TOXINA	MÉTODO	FUENTE	REFERENCIA
SEA-SED	The optimum sensitivy plate (OSP) method	Productos lácteos	Veras *et al.,* 2008
SEB	Test immunocromatográfico	Patrón de SEB	Khreich, 2008
SEA	Nano LC-MS-QTOF	Patrón de SEA	Dupuis *et al.,* 2008
SEB	Separación por partículas magnéticas / MALDI-TOF MS	Patrón de SEB	Schlosser *et al,* 2007
SEA	Ensayo cuantitativo con MS usando isótopos marcados específicamente [^{13}C] [^{15}N]	Perlas de coco	Hannekinne *et al.,* 2009
SEB	LC/MS-MS	Apple juice	Callahan *et al.,* 2006
SEA	Electroforesis monodimensional / MALDI-TOF	Leche	Sospedra *et al.* 2011
TSST-1	TST-RPLA kit	Alimentos manipulados manualmente	Fueyo *et al.,* 2005b
TSST-1	TST-RPLA kit	Alimentos listos para tomar	Su *et al.,* 2007
TSST-1	TST-RPLA kit	Muestras clínicas y de alimentos	El-Ghodban *et al.*, 2006
TSST-1	TST-RPLA kit	Portadores nasales	Fueyo *et al.,* 2005a
TSST-1	TST-RPLA kit	Muestras clínicas de recién nacidos hospitalizados	Cunha *et al.,* 2007

OBJECTIVES

The **overall objective** of this study is to conduct the assessment and monitoring of those, microbiological and toxicological parameters that, directly or indirectly may affect the quality of processed foods or consumers´ health.

To achieve this general objective the following specific objectives have been set:

1. To study the quality assessment of frying oils used in the university food services.

2. Assessment of anisakis contamination in fish based meals served at university restaurants.

3. To assess the microbiological quality of the menus offered in the university food services.

4. Application of chromatographic techniques for detection and quantification of microbial toxins.

OBJETIVOS

El **objetivo general** del presente trabajo es realizar la valoración y seguimiento de diversos parámetros microbiológicos y toxicológicos que, directa o indirectamente pueden afectar a la calidad de los alimentos elaborados y a la salud de los consumidores.

Para alcanzar este objetivo general se plantean los siguientes objetivos específicos:

1. Evaluación de la calidad de los aceites de fritura utilizados en los servicios de restauración.

2. Estudio de la presencia de anisakis en los platos preparados a base de pescado servidos en los servicios de restauración universitarios.

3. Evaluación del estado microbiológico de los alimentos ofertados en los menús de los servicios de restauración.

4. Aplicación de técnicas cromatográficas a la detección y cuantificación de toxinas microbianas.

WORKING PLAN

To achieve the objectives proposed a working plan has been designed with the following steps:

1. To study the quality assessment of frying oils used in the university food services, frying oil samples were collected from university establishments and analyzed, according to current legislation, for the determination of total polar compounds. According to results obtained, oils were classified as acceptable or unacceptable to be hazardous to consumers´ health.

2. To assess contamination of anisakis in fish based meals served at university restaurants. Fish consumed at university establishments undercooked or without prior freezing were studied for the presence of anisakid parasites. The analysis is performed by digestion technique.

3. In order to evaluate the microbiological quality of the menus offered in the university food services, microbial flora present in the menus was determined over a 3 years period.
   Food samples of most consumed dishes were collected from university restaurants and analyzed according to the current legislation by classic microbiological methods.

4. To evaluate the incidence of TSS toxin from *S. aureus* in kitchen surfaces and foodhandlers an immunological technique (TSST-RPLA) was been employed.
5. To identify and quantify Staphylococcal and *E. coli* toxins most commonly involved in food poisoning, several chromatographic techniques were developed.

   Subunit B from heat labile enterotoxin produced by *E. coli* was characterized by liquid chromatography-mass spectrometry (LC-MS), MALDI-TOF and liquid chromatography-diode array detection (LC-DAD) techniques.

   Staphylococcal enterotoxins A and B were also identificated or quantified by these techniques. The presence of these toxins was also investigated in bacteria cultures from strains isolated from food samples collected at university restaurants.

EXPERIMENTAL SECTION

1. TOXICOLOGICAL-CHEMISTRY ASPECTS OF FRYING OILS FROM UNIVERSITY RESTAURANTS

1.1. Introduction

Deep-fat frying is, worldwide, one of the most used cooking methods (Blumenthal *et al.,* 1991). Especially French fries are appreciated by consumers, at home and in restaurants, due to its pleasant taste, flavors and crispy crust (Ross *et al.,* 2004). In Spain, consumption of French fries is around 2.7 Kg/person/year, and mainly sunflower oil is used to cook this product in foodservice establishments (Romero *et al.,* 2000; Soriano *et al.,* 2002c). The fat content of French fries is likely to contribute to obesity by increasing the intake of total fat and energy. This oil can also be a health hazard because during the frying process, the sunflower oil is subject to several changes including reactions which produce compounds that are harmful to human health (Dobarganes and Márquez Ruíz, 2007). The quality of frying oil is important due to absorption into the food during frying, thereby affecting the quality of the final product (Orthoefer *et al.,* 1996). For this reason, monitoring the quality of the frying oil is an important tool to guarantee the food safety. The measurement of the total polar compounds is the most reliable method standardized and regulated in many nations where any frying oil with a concentration of total polar materials below the specified limit, say 25% by mass, is considered chemically good and acceptable. This means that any batch of oil can be maintained below this

concentration by regular replenishment, without replacing the entire batch of oil (Gupta, 2004). The aim of this part of the dissertation thesis is to evaluate, during four years (2008-2011), the quality of sunflower oil used in frying processes by monitoring the presence of polar compounds in oil/fat used at university restaurants.

1.2. Material and methods

1.2.1. Samples and sampling

Sunflower oil samples used in frying were taken from 23 university restaurants belonging to five different food service companies. All sampled restaurants reported to use sunflower oil as frying oil. Samples were taken from fryers when the oil was in use. Inspections at food service establishments and sample collection was done randomly during 4 years, two or three times a year, depending on the availability of restaurants. According to several criterions admitted in most of the European countries (Paul *et al.,* 1997), if any of the following conditions are not satisfied; i) frying fats and oils must be organoleptically acceptable, ii) the polar compounds should not exceed 25 % by mass, iii) frying fats must not alter the quality of fried food and iv) frying fat must not be sold for subsequent use in preparing other food products after it has been used in frying operations, oil must be discarded. To check if these conditions are met, samples were taken from electric fryers used for frying of French fries and the temperature was measured in the frying process with a Crison 638 Pt digital

thermometer (Crison Instruments, Barcelona, Spain). Samples of 25 g of frying oil were then placed into glass jars, transported to the laboratory and stored at -20 ºC in the dark before analysis to prevent further oxidation.

1.2.2. Polar compounds analysis

Oil analysis was performed, according to IUPAC Method (1987), in a glass column packed with silica gel. Using a mixture of light petroleum ether and diethyl ether (87:13) the polar and the nonpolar parts of the frying fats and oils can be separated. Unchanged nonpolar triglycerides are eluted by this mobile phase, while polar substances are adsorbed on the silica gel. For the elution of the polar compounds pure diethyl ether has to be used. The amount of each fraction has to be determined by weight after evaporating the solvent.

1.3. Results and discussion

Table 14 shows the incidence of acceptable and unacceptable frying oils with more than 25% of polar compounds. All samples, at 2011, had values indicating of adequate amount of polar compounds. However, along the previous years, (2008 to 2010), it was observed the presence of some samples with unacceptable levels. According to our results, several previous studies in fast-food restaurants, reported oil samples of frying oil with total polar compounds at unacceptable levels, being the percentage of samples with the highest value in Finland (60%) (Skrokki, 1995),

followed by France (48.4%) (Sebedio *et al.,* 1987), Sweden (38.0%) (Croon *et al.,* 1986), Germany (35.2%) (Gertz, 1986), Spain (34.5%) (Dobarganes *et al.,* 1995), Brazil (30.0%) (Ans *et al.,* 1999) and Turkey (32% and 10.7%) (Yilmaz *et al.,* 2011; Hampikyan *et al.,* 2011). In our study, an improvement of the quality of the oils is observed over the four years of sampling. The reduction of the polar compounds was observed in line with the internal monitoring of the foodservice establishments next to the use compulsory, from 2010, of rapid tests for monitoring oil quality by chefs and food handlers in these establishments (personal communication obtained from restaurateurs). All restaurants sampled have implemented in last year an internal control planning and are working with Fritest® as rapid colorimetric test which is used by restaurateurs in several foodservice establishments and by the food inspectors on routine inspections (Stier, 2004; Firestone, 2007; White, 1991). This fact is supported by Soriano *et al.* (2002c) and Vorria *et al.* (2004), who demonstrated the efficiency of the HACCP system applied in frying oils which is enforced in Europe, according to the the Directive 93/43/EC and Regulation 178/2002/EC, to guarantee food hygiene and safety of the food production chain, respectively.

On the other hand, the temperature of frying oil measured, in our study, reflected a range from 170 to 190 ºC with a mean temperature (SD) of 185.1 ºC (12.1) being these values similar to other authors (Soriano *et al.* 2002c; Vorria *et al.* 2004, Gertz, 2000).

Table 14. Incidence of acceptable and unacceptable frying sunflower oils from university restaurants.

Food service establishment	2008			2009			2010			2011		
	≤20%	20-25%	≥25%	≤20%	20-25%	≥25%	≤20%	20-25%	≥25%	≤20%	20-25%	≥25%
1	1/3	1/3	1/3	2/3	0/3	1/3	0/2	2/2	0/2		n.c.	
2	0/3	2/3	1/3	2/3	1/3	0/3	3/3	0/3	0/3	1/2	1/2	0/2
3	0/3	1/3	2/3	0/3	0/3	3/3	1/3	2/3	0/3	2/2	0/2	0/2
4	1/3	1/3	1/3	2/3	0/3	1/3	2/2	0/2	0/2		n.c.	
5	0/3	1/3	2/3	0/3	2/3	1/3	1/3	2/3	0/3	0/2	2/2	0/2
6	0/3	1/3	2/3	1/3	0/3	2/3	0/3	2/3	1/3	1/2	1/2	0/2
7	2/3	1/3	0/3	3/3	0/3	0/3	3/3	0/3	0/3	2/2	0/2	0/2
8	2/3	0/3	1/3	1/3	0/3	2/3	3/3	0/3	0/3	2/2	0/2	0/2
9	0/3	2/3	1/3	0/3	2/3	1/3	2/3	1/3	0/3	2/2	0/2	0/2
10	0/3	0/3	3/3	1/3	0/3	2/3	0/3	2/3	1/3	0/2	2/2	0/2
11	1/3	2/3	0/3	3/3	0/3	0/3	3/3	0/3	0/3	2/2	0/2	0/2
12	1/3	1/3	1/3	0/3	2/3	1/3	0/3	3/3	0/3	1/2	1/2	0/2
13	0/3	2/3	1/3	1/3	1/3	1/3	3/3	0/3	0/3	2/2	0/2	0/2
14	3/3	0/3	0/3	3/3	0/3	0/3	3/3	0/3	0/3	2/2	0/2	0/2
15	0/3	2/3	1/3	2/3	1/3	0/3	2/3	1/3	0/3	2/2	0/2	0/2
16	1/3	0/3	2/3	2/3	0/3	1/3	3/3	0/3	0/3	2/2	0/2	0/2
17	1/3	1/3	1/3	2/3	0/3	1/3	3/3	0/3	0/3	2/2	0/2	0/2
18	0/3	2/3	1/3	2/3	0/3	1/3	1/3	2/3	0/3	2/2	0/2	0/2
19	1/3	2/3	0/3	3/3	0/3	0/3	3/3	0/3	0/3	2/2	0/2	0/2
20	2/3	0/3	1/3	2/3	0/3	1/3	2/3	1/3	0/3	2/2	0/2	0/2
21	1/3	1/3	1/3	2/3	0/3	1/3	2/3	1/3	0/3	2/2	0/2	0/2
22	0/3	2/3	1/3		n.c.			n.c.			n.c.	
23		n.c.			n.c.		0/1	1/1	0/1	2/2	0/2	0/2

n.c.: not collected. No samples were collected in this period because the food service establishment was not in use.

If French fries are cooked at lower temperature, the crust does not form on the surface. This allows extra fat to penetrate into the core of the French fries. About 40% more fat is absorbed when oil temperature is 10°C lower than the recommended cooking temperature of 180°C to 185°C (Mehta *et al.,* 2001). Morley-John *et al.* (2002) demonstrated a wide range of measured fat temperature (136-233°C) with a mean temperature (SD) of 181.9°C (15.3) in fast food outlets in New Zealand. They reported that 44% of the independent outlets had thermostats that were unreadable or inaccurate. The same problem was observed, in our study, from 2008 to 2009 in nine restaurants. However, all thermostats were repaired or changed in the studied last two years and it help to adjust the maximum rate of fat turnover and remove food particles from the fat.

Internal control of polar compounds, along with a correct measurement of temperatures allows keeping the oxidation level of the frying fat to a minimum as guarantee of a good-quality of the oil.

To decrease the excessive oxidation of frying oil and to satisfy the critical limits for the frying temperature and the potential hazards, some important factors must be monitored. The control of the initial oxidative state of the oil/fat used, the temperature of frying and the renewal of the oil/fat during frying can help to the reduction of polymerization and formation of various decomposition products as are volatile (peroxides, monoglycerides, diglycerides, aldehydes, ketones and carboxylic

acids) and non-volatile decomposition products (cyclic monomers, dimmers, trimers, other high molecular-weight compounds) include polar compounds which may result in gumming and foaming.

1.4. Conclusions

In conclusion, training personnel, HACCP application and routine inspections contribute to improve frying process to guarantee both hygiene and safety of the food along the production chain. In the light of the results obtained in this study, we will carry out, in next years, a program to obtain the certification of operators for deep frying according to food service personnel and restaurateurs, similar to certification for food safety, as another option for promoting best practice deep frying techniques as it was observed in the study of Morley-John *et al.* (2002).

2. ABSENCE OF PARASITES (*Anisakis simplex*) IN FISH FROM UNIVERSITY RESTAURANTS

2.1. Introduction

The University restaurants offer fish as the fourth most consumed food in comparison with cereals, vegetables, and meat dishes. This food is responsible, in Spain, for several hundred cases of allergy to *Anisakis simplex* in the last few years (Fernández de Corres *et al.,* 1996, Montoro *et al.,* 1997, Fraj Lázaro *et al.,* 1998) due to consumption of undercooked fish and/or mainly homemade anchovies in vinegar, homemade without prior freezing. The aim of this part of the disseration thesis is to evaluate the presence of anisakid parasites in raw seafood dishes from University restaurants.

2.2. Material and methods

2.2.1. Samples and sampling

A total of 54 samples of raw *Engraulis encrasicholus* (anchovies) were collected aseptically in sterile bottles (VWR International Eurolab, Barcelona, Spain) from 2008 to 2011. The family *Engrauldae* includes some of the most consumed genus as *Engraulis encrasicholus,* usually eaten like white or red anchovies. Usually white ones are prepared in vinegar and are called (*boquerones*). Samples were kept under refrigeration between 0 and 4 ºC up to the time of the parasitological analysis. Furthermore, all establishments were asked about the origin and

food preservation method of the fish served in restaurants, and although all restaurateurs claimed that mainly all the fish was bought or conserved freeze, some samples were bought as fresh fish and conserved in vinegar. To assess the parasitic quality and verify the application of good practices in the treatment of fish samples the analysis to determine the presence or absence of *Anisakis simplex* was carried out.

2.2.2. Analysis of anisakid parasites

The artificial digestion method described by Huang (1990) was employed to determine the presence of anisakid nematodes.

The flesh from each sample was examined, i.e. it was subjected to artificial digestion in an aqueous HCl-Pepsin solution. The procedure was conducted by using 1 L glass flasks each containing 5 g of commercially available pepsin (proteolytic activity 1:2,500), 10 mL of HCl and 1 L of saline solution. Digestion flasks were placed in a water bath of 37 °C and shaken mechanically at 250 rpm for 45 minutes.

After digestion, the solution was sieved (1.5x1mm mesh), and the solid residues were examined under a binocular microscope (10-40 fold magnification).

2.3. Results and discussion

Usually restaurants buy frozen fish, and this treatment ensures the absence of live anisakis. Recommendations are established in Spain (Anonymous, 2006) and in the European Union (1991, 2004),

and according to it, to prevent the infestation of the larvae by the consumer, in many countries, all establishments where fish is served are required to ensure that fishery products for raw consumption are processed by mild treatments that do not kill the parasite have been previously frozen at or below -20 ºC in all parts of the product for a period of at least 24 hours. Only 2 out 23 restaurants included in our study bough fresh fish. The genus bought as fresh fish was *Engraulis encrasicholus* and it was prepared at restaurants to be served with vinegar and spices. A recent study concluded that marinated fish treatment using low pH to enlarge the storage life of fish as in anchovies in vinegar, does not kill Anisakis simplex larvae infesting fish muscle (Solas *et al.,* 2009).

In our study, no fish sample had evidence of anisakid parasites, although in Spain, several hundred cases of allergy to *A. simplex* have been reported in the last few years (Fernández de Corres *et al.*, 1996; Montoro *et al.*, 1997; Fraj Lázaro *et al.*, 1998) due to consumption of undercooked fish and/or mainly homemade white anchovies in vinegar, without prior freezing. Furthermore, there is an evidence of a marked variation in *A. simplex* infection risk among the different Spanish regions, ranging from 0.4% in Galicia (N.W. Spain) (Valiñas *et al.*, 2001) to 15.7% and 22.1% inland and southern regions, respectively (Fernández de Corres *et al.,* 2001; Del Rey Moreno *et al.*, 2006; Puente *et al.*, 2008). Lunestad (2003) examined 1.180 samples of muscle or viscera from Norwegian-farmed salmon and none of the

samples contained nematodes. Moreover, some recent studies revealed high prevalences of anisakids in commercialized anchovies in Spain, ranging from 23 to 32 % (Rello-Yubero *et al.* 2009). Fortunately, our study also reflected the absence of this parasite in studied fish samples.

2.4. Conclusions

In conclusion, our study reflected the absence of this parasite in studied fish samples due to the implementation of the Spanish Royal Decree 1420/2006 on prevention of parasitosis by *Anisakis* legally bound to freezing (at -20 C for 24 h) and thorough heating (to at least 60 ºC for at least 1 min) to guarantee the destruction of parasite larvae (Domínguez and Martínez, 2000). These procedures must be supervised continuously to improve the quality of raw and prepared seafood dishes and consequently to protect consumers' health.

3. MICROBIOLOGIAL ASPECTS OF THE READY-TO-EAT DISHES SERVED IN UNIVERSITY RESTAURANTS

3.1. Introduction

Foodborne disease has emerged as an important and growing public health and economic problem in many countries during the last decades. A wide range of foods can be involved as sources of foodborne diseases in restaurants due to incorrect food processing operations. The infection agent may be transferred to food directly or by cross-contamination, so hygienic food preparation and the education of those involved in preparation, processing and serving of meals are crucial lines of defense in the prevention of most types of foodborne illness. Microbiological control allows for identifying, evaluating and preventing contamination risks to the end consumer, through food products, and it is essential to guarantee food safety. According to the results obtained after nutritional evaluation and the eating pattern of university students, ready-to-eat foods have been classified in different groups attending to their most caloric contribution to consumers' diet and their main and more representative component. The aim of this part of dissertation thesis is to evaluate, in university establishments, the microbial quality of dishes and beverages of the most consumed products in these establishments.

3.2. Material and methods

3.2.1. Food samples

Food samples were purchased over a period of three years from University restaurants in Valencia (Spain). Hygienic and unhygienic practices in the preparation of these foods were also studied according to Soriano *et al.* (2001c). Analyzed products include raw and processed foods that are commonly consumed in these establishments (Mendonça *et al.,* 2011). Immediately after collection, samples were chilled to 4 ºC and transported to the laboratory for analysis. The microbiological analysis was done on the same day.

Foods analyzed were divided in 8 groups according to their major component:

a) Cereals

b) Legumes

c) Vegetables

d) Meat

e) Fish

f) Milk and dairy products

g) Fruits and fruit juices

h) Eggs and egg products

3.2.2. Sampling plan

A total of 2116 lots were collected randomly and aseptically in sterile bags and bottles (VWR International Eurolab, Barcelona,

Spain). For each sample, five replicates were taken. A fundamental principle of lot acceptance sampling plans is that the samples collected will reflect the lot as a whole. For this reason, it is critical that the samples be collected at various points throughout the entire lot.

When more than one sample is analyzed for a microbiological attribute, a two- or three-class sampling plan may be applied to evaluate results. The sampling plan is a systematic way to assess the microbiological quality of food lots. A "lot" refers to a batch of products manufactured under the same conditions at the same time. During sampling, the samples (abbreviated as "n") should be taken from the lot independently and randomly.

For this work, the two types of sampling plans most commonly used in food microbiology have been applied. The attributes of these sampling plans are given below:

a) Two-Class Sampling Plan.

A two-class sampling plan is appropriate when zero positives are permitted. In a two-class sampling plan, $c^1=0$ and $m^2=M^3$ in that there is no marginal range of acceptance and no sample may contain levels greater than m. In this plan, if all the samples are ≤ m or ≤ c of samples are > m, lots are accepted. However, if > c of samples are within > m, lots are rejected.

b) Three-Class Sampling Plan.

[1] Number of samples that may be tolerated in the marginally acceptable range (area between m and M).
[2] Value below which all values are acceptable.
[3] Value at which all values above are defective.

A three-class sampling plan is appropriate when a proportion of the sampled units may yield test values in a marginally acceptable range without causing consequent problems (ICMSF, 2002). This is often true for testing indicator microorganisms, such as aerobic bacteria and Enterobacteriaceae. It may also be appropriate for certain bacterial pathogens where a tolerance can be established in a product without endangering safety, such as *C. perfringens, B. cereus,* or *S. aureus*.

For a three-class attributes plan, two microbiological limits, m and M (limit beyond which the level of contamination is hazardous or unacceptable), are set and if all the samples are ≤ m or ≤ c of samples are > m, lots are accepted. However, if > c of samples are within > m and < M or any sample is > M, lots are rejected.

3.2.3. Legislation

The microbial quality of the food was evaluated attending to the specifications of the Spanish Regulation No. 3484/2000 (Anonymous, 2001), Commission Regulation No. 2073/2005 (European Union, 2005), Commission Regulation No. 1441/2007 (European Union, 2007) and Commission Regulation No. 365/2010 (European Union, 2010). According to these legislations and ICMSF (1986), two-class attributes plan is preferred when the microorganism of concern is not permitted in the food (*Listeria monocytogenes*, and *Salmonella* spp.). However, if the number of

microorganisms must be counted (aerobic plate counts, Enterobacteriaceae, *Staphylococcus aureus, Escherichia coli*), a three-class attributes plan is adopted.

3.2.4. Microorganisms analyzed

According to legislation cited before, microorganisms monitored to assess the quality of food served at university are:

-Total count of aerobic mesophilic bacteria.

-Total count of Enterobacteriaceae.

-Total count and identification of *S. aureus*.

-Total count and identification of *E. coli*.

-Isolation and identification of *Salmonella* spp.

-Isolation and identification of *L. monocytogenes*.

3.2.5. Microbiological analyses

Twenty five grams or twenty five milliliters of each of the samples collected were suspended in 225 ml of buffered peptone water (BPW) (Oxoid, Unipath, Hampshire, United Kingdom) and aseptically homogenized in a stomacher (Classic; IUL, Barcelona, Spain). Serial decimal dilutions were prepared from this initial dilution. Each of the different dilutions (1 ml or 0.1 ml) was transferred onto the surface of the plates containing appropriate cultures media for each microorganism analyzed.

a) Aerobic mesophilic bacteria.

Four 10-fold dilutions were done with each sample, and 1mL of each step was inoculated onto duplicate plates of standard

Plate Cont Agar (Oxoid) and incubated at 30 ºC ± 1 ºC for 72 ± 3 hours, according to the ISO 4833 reference method (International Organization for Standardization, 2003). To determine the total aerobic mesophilic bacteria, the three-class sampling plan was the sampling plan selected.

b) Enterobacteriaceae.

According to the ISO 21528-2 (International Organization for Standardization, 2004), duplicate poured plates of Violet Red Bile Glucose agar (Oxoid), were inoculated with four different decimal dilutions of each sample. The plates were incubated at 37 ºC for 24 hours, and typical colonies were counted on all plates according to the three-class sampling plan established and having not more than 150 typical colonies.

c) *Staphylococcus aureus.*

For enumeration of *S. aureus*, 0.1 ml of the inoculated BPW was surface plated on Baird Parker (BP) agar containing egg yolk tellurite emulsion (Oxoid) and incubated at 37 ºC ± 1 ºC for 24 + 24 hours (International Organization for Standardization, 1999). Typical colonies (i.e., black, convex and with or without halo on BP agar) were counted, examined microscopically, tested for catalase reaction, and confirmed with the agglutination Staphytect Plus test (Oxoid).

d) *Escherichia coli.*

Attending to ISO 16649-2 (International Organization for Standardization, 2001), to isolate *E. coli*, the previous BPW tubes were inoculated onto CHROMagar ECC (CHROMagar Microbiology, Paris, France). After incubation at 37 ºC for 48 hours, colonies were confirmed using Rapid ONE System (Remel Inc., Lenexa, KS).

a) *Salmonella* spp.

Isolation and identification of *Salmonella* spp. was done according to the ISO 6579 (International Organization for Standardization, 2002) and performed using the homogenate in BPW. Quantities of 1 and 0.1 ml of the BPW were inoculated into Tetrathionate broth with Novobiocin (Oxoid) and Rappaport-Vassiliadis broth (Oxoid), respectively. The enrichment broths were incubated for 24 ± 2 hours respectively at 37 ºC ± 1 ºC (for Tetrathionate broth with Novobiocin) and 42 ºC (for Rappaport-Vassiliadis broth). The positive cultures were streaked onto XLD *Salmonella* agar (Oxoid) at 37 ºC ± 1 ºC for 24 hours, and the confirmation was done using the Rapid ONE System (Remel Inc.).

b) *Listeria monocytogenes.*

L. monocytogenes was enumerated according to the ISO 11290-1 (International Organization for Standardization, 1996). Samples (25 g) were weighed into sterile stomacher bags,

diluted, and homogenized with 225 ml of Fraser broth (Oxoid). After homogenizing and preculturing at 37 ºC ± 1 ºC for 48 ± 2 hours, the positive broth was streaked onto Listeria Palcam agar (Oxoid) and incubated at 37 ºC ± 1 ºC for 24 ± 2 hours. Characteristic colonies were Gram stained, tested for motility, oxidase, and catalase followed by identification with the API *Listeria* system (BioMérieux, Mancy l'Etoile, France).

3.3. Results and discussion

3.3.1. Cereals

All samples collected were divided into 2 types, wheat and rice-based dishes, according to their main component.

Results obtained reflect that only 12 out 102 wheat samples were above legal limits for aerobic colony count, however, for Enterobacteriaceae, 40 wheat-based dishes presented an unacceptable quality (Table 15). There are few reported incidents of food poisoning resulting from contaminated cereals. Unprocessed products (grains and flours) may contain high bacteria levels, however this contamination is low in processed and end products (Berghofer *et al.,* 2003). The process of cooking should kill the bacteria but some bad practices of handling or storage can even increase the bacterial load of the initial product.

Most wheat samples were pasta dishes cooked as salads and some with sauces as meat with tomato or with milk cream. Macaroni and spaghetti with tomato sauce were the pasta dishes most consumed and also most contaminated (8 out 12

unacceptable for total aerobic mesophilic bacteria and 23 out 40 for Enterobacteriaceae), followed by pasta with milk cream and salad pasta.

Table 15. Microbial quality, according to the European and Spanish legislations, for total aerobic mesophilic bacteria and Enterobacteriaceae in cereal dish samples collected from studied foodservice establishments.

	Microbial quality (%)					
	Total aerobic mesophilic bacteria			Enterobacteriaceae		
Source (no. of lots)	Acceptable	Marginal	Unacceptable	Acceptable	Marginal	Unacceptable
Wheat (n=102)	71	18	11	58	3	39
Rice (n=59)	49	24	27	85	2	13

Our results are according to other studies about ready-to-eat pasta dishes (Yeboah-Manu *et al.,* 2010; Mensah *et al.,* 2002). Yeboah-Manu *et al.* (2010) studied the bacterial quality of ready-to-eat foods sold on and around the university of Ghana campus. They found macaroni samples had very high levels of bacterium. This may be due to cooking process because macaroni is prepared by boiling it in hot water for a few minutes and draining the water from it. Though the water is drained from the food, it still has a moist appearance and this provides a good environment for bacterial growth. If pasta dishes are mixed with tomato or milk

cream after cooking, these sauces can make it very rich and could also account for the level of contamination.

Examination of cooked rice revealed that the majority (85%; 50 of 59) had a satisfactory/acceptable microbiological quality whereas only 8 (13%) had an unsatisfactory microbiological quality for Enterobacteriaceae analysis. However, 27% of all the rice lots studied exceeded the European maximum level of mesophilic aerobic counts (Table 15). Little *et al.* (2002) also found high levels of aerobic colony counts in cooked rice.

The most common ways of cooking rice in studied restaurants are all the variants of *paella* (a traditional Spanish one-pan meal made with rice as the principal ingredient), however none of these samples presented high levels of microbial contamination. More than 90% of unacceptable samples were rice salads. Many vegetables used for salads are not cooked before eating and it is common practice for raw vegetables to be washed, chopped, packaged, chilled or frozen before consumption in ready-to-eat salads. Some of these vegetables can contribute to rice-based dishes contamination as a result of handling involved in preparation, the manufacturing processes, and temperature control of the final product. This fact is also supported by other studies like the work of Little *et al.* (2002) who established that microbial quality of cooked rice was significantly associated with cooking, serving methods and management food hygiene training.

Although several pathogens microorganism have been implicated in outbreaks caused by dry foods lately, with ready-to-

eat cereal products having the greatest relevance (CDC, 1998; Breuer, 1999), in our study no sample collected was contaminated by *E. coli*, *S. aureus*, *Salmonella* spp. neither *L. monocytogenes*.

3.3.2. Legumes

Three different kinds of legume-based traditional dishes were collected from restaurants and the results of microbial analyses are summarized in Table 16.

Twelve percent of the samples analyzed contained levels of aerobic mesophilic bacteria or Enterobacteriaceae at unsatisfactory levels according to current European legislation.

Results obtained are at odds with previous studies about microbial quality of legume-based foods in which the percentage of contaminated samples by aerobic mesophilic bacteria is higher than ours (Roy *et al.,* 2007; Saroj *et al.,* 2006).

All kinds of legume-based dishes presented contamination by Enterobacteriaceae but chickpeas samples had the highest percentage (25%). Enterobacteriaceae counts are considered more generally as an indicator of hygienic quality rather than of faecal contamination, so the occurrence of these microorganisms in a food is considered as a reflection of the process practised during its preparation and/or subsequent handling under inefficient hygienic conditions (ICMSF, 1978).

In our study, although Enterobacteriaceae occurred in all the three types of studied foods none pathogenic organisms were isolated. Neither *E. coli*, *S. aureus*, *Salmonella* spp., nor *L.*

monocytogenes were detected in legume-based dishes. However, in the work of Saroj *et al.* (2006), *E. coli* was detected in 13% and 26% of two different spices of beans and in 40% of chickpeas. *Salmonella* Typhimurium was detected in 21, 40, and 4% of the above cited samples, respectively.

Table 16. Microbial quality, according to the European and Spanish legislations, for total aerobic mesophilic bacteria and Enterobacteriaceae in legume samples collected from studied foodservice establishments.

	Microbial quality (%)					
	Total aerobic mesophilic bacteria			Enterobacteriaceae		
Source (no. of lots)	Acceptable	Marginal	Unacceptable	Acceptable	Marginal	Unacceptable
Beans (n=14)	79	7	14	86	7	7
Lentils (n=21)	86	9	5	95	0	5
Chickpeas (n=8)	87	13	0	75	0	25

Usually legume-based dishes are meals that do not need manual handling after cooking process so the detection of microorganisms in cooked food samples could be as a result of inadequate cooking, use of unclean utensils and/or recontamination.

According to other studies (ACMSF, 2000; McMeekin *et al.,* 1997) about microbial quality of ready-to-eat foods, post-preparative storage conditions are an inappropriate temperature for food storage or a longer length of time between preparation

and consumption are critical control points that can contribute to food contamination.

3.3.3. Vegetables

Out of 555 samples analyzed, about 13% had unsatisfactory levels for total aerobic mesophilic bacteria and 23% for Enterobacteriaeae microorganisms (Table 17).

Heat-treated foods generally have a good safety record, cooked foods are subjected to heat treatment and when these processes are properly applied, heat can eliminate biological agents that spoil or compromise food safety. The applied treatment factors (time/temperature regime) can vary to accomplish almost any degree of microbial inactivation, ranging from limited reductions of microbial load to complete sterilization (Rajkovic *et al.,* 2010; Juneja and Novak*,* 2003; Yousef et al., 2003). In our study, all samples collected were from ready to eat dishes; some of them had been cooked previously but, in salad dishes, ingredients are eaten as fresh vegetables without cooking processes.

Lettuce and tomato samples were collected as raw and fresh vegetables, for this reason results that show both vegetables as more contaminated samples for total aerobic mesophilic bacteria and Enterobacteriaceae were expected. However, despite this expected frequency of contamination, high levels of microorganisms found indicate poor handling practices.

Table 17. Microbial quality, according to the European and Spanish legislations, for total aerobic mesophilic bacteria and Enterobacteriaceae in vegetable samples collected from studied foodservice establishments.

	Microbial quality (%)					
	Total aerobic mesophilic bacteria			Enterobacteriaceae		
Source (no. of lots)	Acceptable	Marginal	Unacceptable	Acceptable	Marginal	Unacceptable
Lettuce (n=137)	70	10	20	47	9	44
Tomato (n=77)	80	5	15	69	5	26
French beans (n=72)	89	3	8	89	5	5
Potatoes (n=80)	86	3	11	79	1	20
Zucchini (n=44)	91	7	2	86	5	9
Pepper (n=30)	73	17	10	73	0	27
Green peas (n=28)	96	0	4	100	0	0
Cauliflower(n=21)	90	10	0	86	14	0
Spinaches (n=11)	82	9	9	64	0	36
Broccoli (n=10)	30	20	50	30	0	70
Eggplant (n=10)	60	0	40	80	0	20
Mushrooms (n=10)	90	10	0	90	0	10
Onion (n=9)	78	11	11	67	0	33
Lima beans (n=6)	83	0	17	83	0	17
Artichokes (n=5)	100	0	0	100	0	0
Corn (n=5)	100	0	0	0	40	60

According to the results reported by several reviews (Little and Guillespie, 2008; Heaton and Jones, 2008) consumption of fresh fruits and vegetables could be a risk factor for infection with

enteric pathogens. Recent examples of outbreaks related to fresh produce include cases of *E. coli* (spinach, lettuce) and many species of *Salmonella*. In 2008 a nationwide outbreak of *Salmonella* Enterica occurred in Finland. A total of 77 culture-confirmed *Salmonella* Newport and 30 *Salmonella* Reading cases were reported. The trace back investigation suggested that the factor connecting the cases was ready-chopped iceberg lettuce available for mass catering use (Lienemann *et al.,* 2011).

Out of 555 vegetables only one of 137 lettuce samples was contaminated by *Salmonella* and *E. coli* but fortunately, no cases of foodborne diseases were reported (Table 18).

Table 18. Pathogenic microorganisms in vegetable dishes collected from studied foodservice establishments.

	LETTUCE (n=137)	FRENCH BEANS (n=72)	POTATOS (n=80)	OTHERS (n=4)
Staphylococcus aureus	8 %	2.8 %	1.2 %	n.d.
Escherichia coli	6.6 %	n.d.	n.d.	n.d.
Salmonella spp.	0.7 %	n.d.	n.d.	n.d.
Listeria monocytogenes	n.d.	n.d.	n.d.	n.d.

n.d.: not detected

Human listeriosis disease is largely attributed to foodborne transmission of the microorganism. *Listeria* is a pathogen often isolated in raw vegetables and it has been found in samples collected from Spanish restaurants. Soriano *et al.* (2001a) isolated different *Listeria* species from ready-to-eat lettuce samples from

food establishments. Our study does not reflect any evidence of *Listeria* in vegetables studied.

In the harvest, transport and/or storage of foods, bacteria can adhere and also propagate on the surface of the foods. Such bacteria will adversely affect consumers´ health and therefore should also be removed from the surface of foods before ingesting. It has long been a practice to wash foods such as vegetables and fruits with chlorinated water, a saline solution or appropriate detergents to remove bacteria. In restaurant establishments studied, 14 samples, including both cooked and fresh vegetables (11 lettuce, 2 French beans and 1 potato) were found to be contaminated by *S. aureus*.

In literature, some comparisons between raw and cleaned vegetables can be found. Soriano *et al.* (2001b) studied microbial contamination in raw and `ready-to-eat' lettuces and they found the same percentage of samples contaminated by *S. aureus* in both products. Ready-to-eat salads have been previously cleaned and this means that contamination might be caused by incorrect cleaning practices or inadequate hygienic handling. Our lettuce samples could be contaminated by these reasons too. Considering that French beans and potato meals are cooked dishes, it is more likely that contamination occurred after food processing. Cross contamination is reported as a contributory factor outbreak associated with the consumption of fruits and vegetables. Infected food handlers are often implicated in outbreaks of known or suspected viral or bacterial etiology and might well have been the

cause of many of these outbreaks. There are some cases of food borne diseases in which the food handling faults have contributed to food contamination. It is recorded in one outbreaks report from England and Wales that a restaurant chef was suddenly taken ill while preparing a meal. He vomited over the salad he was preparing. He rinsed the salad in cold water and it was then served to the customers. Many of these customers subsequently became ill. In other outbreak cause by *E. coli*, restaurant staff revealed that the salad items were stored in plastic containers prior to serving. These containers were also used for storing raw beef and were rinsed out before re-using for salad vegetables (ACMSF, 2000).

3.3.4. Fruits and fruit juices

To prevent or reduce risk of foodborne illness or injection by contaminated fruits an important step is to wash all raw fruits before serving or combining with other ingredients. Usually, fresh fruit are served whole and uncut. For this reason unpeeled fresh fruit has a smaller microbial risk than fruits served cut into pieces or fruits that have undergone heat treatment or other processing to make other products such as juices.

All samples collected were peeled or cut fruit and also fruits treated to cooking were analyzed.

“Cut product” is defined as "Any product where the intact protective surfaces of the plant have been breached or removed". The process of cutting can have some potential consequences; it

can remove the pathogen if it is present on the outside of the fruit but only if the process is carried out in a proper hygienic manner.

It can give the pathogen access to the nutrients available on and from the inside of the fruit. This can lead to multiplication of certain pathogens during storage. It can spread the pathogen from contaminated to uncontaminated product as a result of inappropriate hygiene of large batches of the product during processing (ACMSF, 2000). Our results reflect a good fruit quality for aerobic colony count. We have not found any fruit sample contaminated at unacceptable levels by these microorganisms (Table 19). However, some samples exceeded legislated levels for Enterobacteriaceae. Although some reports about outbreaks associated with fruit products have shown that fruits such as melon or cantaloupe can act as a vehicle for food-borne pathogens as *Salmonella*. (CDC, 2002; Institute of Food Technologists, 2001; Sivaplasingham *et al.,* 2004; Tauxe *et al.,* 1997), fortunately, none of the fruit samples analyzed was contaminated by pathogens as *E. coli*, *S. aureus*, *Salmonella* spp. or *L. monocytogenes*. Wilson *et al.* (1955) also reported typhoid fever and salmonellosis illness in Australia caused by the ingestion of contaminated coconut. According to them, other authors have also shown coconut as cause of salmonellosis in England (Ward *et al.,* 1999).

To obtain the orange juice from squeezing machines in all studied foodservice establishments, the procedure was the following; i) Valencia and Navel oranges are introduced through

the feeding tube of the machines, ii) the switching button on is turned and iii) freshly squeezed orange juice is collected in a glass or kept in metal jugs below the machines until consumption. All squeezing machines used in these establishments are Auto Orange Juicer Machine OJ2AAP (Frucosol, Calahorra, Spain). The temperature and pH of the orange juice was measured after sampling with a Crison 638 Pt digital thermometer (Crison Instruments, Barcelona, Spain) and a Crison PH-25 Model portable pH Meter (Crison Instruments), respectively. A total of 190 lots of this type of juice were collected.

Table 19. Microbial quality, according to the European and Spanish legislations, for total aerobic mesophilic bacteria and Enterobacteriaceae in fruit samples collected from studied foodservice establishments.

	Microbial quality (%)					
	Total aerobic mesophilic bacteria			Enterobacteriaceae		
Source (no. of lots)	Acceptable	Marginal	Unacceptable	Acceptable	Marginal	Unacceptable
Melon (n=4)	100	0	0	75	0	25
Coconut (n=5)	80	20	0	60	20	20
Baked Apple (n=9)	0	0	0	67	11	22
Others (n=4)	100	0	0	100	0	0

Fresh orange juice was processed by squeezing machines in restaurants. It was never refrigerated, because juices were served in a glass or maintained in metal jug until the consumption. The

average of temperature and pH of all studied orange juice sampled in these establishments was 17.1 ºC and 3.5, respectively. These machines are easily cleaned but they are time-consuming. In fact, we have observed in sampling that some of these machines have scraps of oranges in the internal tubing. For this reason and according to Lakshmanan and Schaffner (2005), the presence of dirty places in orange juice machines is reflected in the formation of biofilms.

Our analysis showed that 2 and 22% of the studied lots for fresh orange juice sampled from the glass exceeded the European and Spanish maximum level of mesophilic aerobic counts and Enterobacteriaceae, respectively (Table 20), whereas a high percentage, 13, 81 and 1% of the lots of orange juice taken in metal jug, exceeded these levels for mesophilic aerobic counts, Enterobacteriaceae and *S. aureus*, respectively (Table 20). The study of Martín-Diana *et al.* (2009) also shows a significant increase of aerobic counts over storage time.

According to Sospedra *et al.,* (2009), the samples obtained from metal jugs had a high level of microbial contamination due to contamination from the environment and/or inadequate hygienic handling and unsanitary conditions ICMSF (1998).

Lakshmanan and Schaffner (2005) studied the total plate counts on beverage dispensers in use in University dining halls as well as on an identical but new beverage dispenser situated in their laboratory being in the first equipment, the microbial counts highest immediately after a beverage had been dispensed and

then declined gradually over time. However, the laboratory-based dispensers were initially low, but increased over time. Furthermore, they observed the relationship between high microbial counts obtained by swabbing the inside of the dispenser tips and the presence of biofilms.

Table 20. Microbial quality, according to the European and Spanish legislations, for total aerobic mesophilic bacteria and Enterobacteriaceae in fresh squeezed orange juice collected from studied foodservice establishments.

	Microbial quality (%)					
	Total aerobic mesophilic bacteria			**Enterobacteriaceae**		
Source (no. of lots)	Acceptable	Marginal	Unacceptable	Acceptable	Marginal	Unacceptable
Glass[a] (n= 163)	95	3	2	64	14	22
Metal jug[b] (n=27)	47	40	13	13	6	81

[a] Fresh orange juice processed by squeezing machines served directly in a glass.
[b] Fresh orange juice processed by squeezing machines maintained in metal jug during one day.

These authors reflected that the use of a higher concentration (100-ppm chlorine solution) of sanitizer may reduce microbial counts on beverage dispenser tips. In our study 12% and 43% of the total examined lots exceed the adopted limits of mesophilic aerobic counts and Enterobacteriaceae, respectively. Piló *et al.* (2009) detected *S. aureus* in two out of twenty orange juice in a range from 2 to 6.5 x 103 157 colony-forming unit (CFU)/ml and

all samples (n=20) were positive for total coliforms and thermotolerant coliforms from 158 < 2.2 to >16.0 most probable number (MPN)/ml and from < 2.2 to 16.0 MPN/ml, respectively. Iha *et al.* (2000) evaluated the physicochemical and hygienic sanitary quality of the fresh and pasteurized orange juices consumed in these cities. One hundred and thirty fresh and thirty-three pasteurized orange juice samples provided by different plants located in the Araraquara and Ribeirao Preto regions (Brazil) were analyzed. The results showed that for fresh juice 63 (48.5%) samples were in disagreement with physicochemical standards established by the legislation, 8 (6.1%) with relation to microscopic and 65 (50.0%) with relation to microbiological analysis. With regard to the pasteurized juice, 3 (9.1%) and 5 (15.1%) samples did not agree with the legislation according to physicochemical analysis and microscopic examination, respectively. Therefore, no pasteurized juice sample was in disagreement with the microbiologic examination. Training and awareness of food handlers at schools is a fundamental condition for avoiding contamination and ensuring the quality and safety of the food produced (Pistore and Gelinskib, 2006).

In our study, *Salmonella* spp. was detected in one lot of orange juice sampled from a metal jug. Jain *et al.,* (2009) found that unpasteurized orange juice was responsible for foodborne salmonellosis in 152 people in six states in the USA. Vojdani *et al.,* (2008) reviewed fruit juice-associated salmonellosis of illness reported, from 1995 to 2005, to the Center for Disease Control

(CDC), being eight linked to orange juice and five outbreaks were caused by *Salmonella* serovars salmonellosis outbreak that occurred during the summer of 1995 among individuals who consumed non-pasteurized orange juice. Furthermore, *Salmonella* cells in juice were associated with population levels of fecal coliforms and *E. coli* above the upper most probable number limits of detection (>110/ml). However, in our study, neither *E. coli* nor *L. monocytogenes* were detected from any of the samples.

3.3.5. Milk and dairy products

Lots taken were divided into three groups:

1. Warm milk: this was milk that had been warmed in metal jugs or that was kept warm in stainless steel thermal flasks.
2. Room-temperature milk: this milk was offered to consumers in metal jugs, brick flasks, or plastic bottles (made of low-density polyethylene) at the coffee counter.
3. Dairy products: including factory-made products as crème caramel and home-cooked products such as custard, mousse, and pudding made with milk.

The analysis showed that 31% and 35% of all the lots studied exceeded the European maximum level of mesophilic aerobic counts and Enterobacteriaceae, respectively (Table 21). This high frequency of lots with elevated levels of contamination indicates contamination from the environment and/or inadequate hygienic handling and unsanitary conditions (ICMSF, 1998). On the other

hand, 2% of all the lots were positive for *E. coli* that may reflect unsatisfactory hygienic conditions of the milk and dairy products.

Fortunately, all the analyzed lots were negative for the presence of the other foodborne pathogens including *S. aureus*, *L. monocytogenes*, and *Salmonella* spp.

Table 21. Microbial quality, according to the European legislation, for total aerobic mesophilic bacteria and Enterobacteriaceae in milk and dairy products collected from restaurants in Spain.

Sample	Source (no. lots)	Microbial quality (%)					
		Total aerobic mesophilic bacteria			Enterobacteriaceae		
		Acceptable	Marginal	Unacceptable	Acceptable	Marginal	Unacceptable
Warm milk	Jug (n=73)	76	3	21	70	0	30
	Thermal flask (n=22)	94	0	6	89	0	11
Room-temperature milk	Jug (n=35)	94	0	6	88	0	12
	Brick (n=25)	98	0	2	90	0	10
	Bottle (n=25)	98	0	2	98	4	2
Dairy products	Custard (n=30)	77	0	23	87	0	13
	Mousse (n=15)	91	0	9	87	0	13
	Pudding (n=15)	85	0	15	91	0	9
	Crème caramel (n=15)	100	0	0	100	0	0

Our results indicated that each type of the three dairy food groups examined showed different levels of contamination. For example, in the warm milk group, possible infringements of hygienic practices in the handling of the thermal flasks and jugs used as containers were observed.

In these restaurants, milk is usually transferred to the thermal flasks after microwave heating or to the serving jugs after warming and frothing via a steam nozzle from a coffee express machine that is inserted into the milk and maintained in the jug. In this regard the importance of applying proper procedures for cleaning these thermal flasks, which should include rinsing with cold water and washing the thermo flask with warm water to remove milk residues from the thermo, is needed from time to time. In addition, with regard to the milk containers, three hygienic malpractices including i) accumulated residues in the steam nozzle from coffee express machine, ii) cleaning procedures that were carried out infrequently, and iii) washing and cleaning procedures of the steam nozzles and the milk jugs using dirty sponges and dish cloths were observed. These methods/procedures used for cleaning can lead to the transfer of bacteria via the contaminated washing-up water or dishes and sponges and contribute to microbial contamination and possible bacterial growth. All these facts have been reported in the literature by many workers such as Scott and Bloomfield (1990), Enriquez *et al.* (1997), Hilton and Austin (2000), and Mattick *et al.* (2003), who reported that dish cloths and sponges were found to transfer large numbers of microorganisms onto a food preparation surface and thus these cloths, due to its microstructure, transfer a significantly greater proportion of its bacterial load than the sponge. In the second group (room-temperature milk), we have also observed potential unhygienic practices based on the cleaning and drying practices

with dirty dish cloths. For serving milk in this way the restaurateur usually buys milk in brick containers and plastic bottles and uses the metal jug to keep the remaining milk from these containers warm. Milk brick containers are opened with clippers. Milk bottles in turn are opened and left standing without resealing the bottle. These procedures increase the hazard of microbial contamination. Consequently we suggested the use of milk bottles instead of bricks since the bottles could be closed and microbial contamination reduced in this way. In our study, the number of positive samples analyzed in dairy products is higher than the UHT milk, due to improper handling of food. De Buyser *et al.* (2001) suggested this option in the foodborne diseases where milk is implicated.

In the third group (dairy products), the crème caramel samples were the only item that presented a satisfactory microbiological quality being the unique factory-made (crème caramel). On the contrary, the other cooking dairy products, such as custard, mousse, and pudding, which were elaborated in the kitchen of the restaurant, were less satisfactory. Our obtained results indicated that custard presented the most unsatisfactory microbiological quality dairy product. In this regard, Reyes *et al.* (2007) suggested that the dairy products that are cooked in restaurant kitchens are usually more contaminated than industrially manufactured dairy products possibly due to the contaminated hands of workers. Our results are in agreement with the findings of Reyes *et al.* (2007) since one of the most critical points in the handling procedure of

custard (not mousse and pudding) is placing the cookie over the top of this dairy product before serving. This may lead to additional microbial contamination of the product. On the other hand, some researchers have observed that cinnamon is added to the custard by some consumers, this may help to eliminate or reduce some microorganisms as *E. coli*, *L. monocytogenes*, and *Salmonella* spp. (Friedman *et al.,* 2002; Cava *et al.,* 2007). Incorrect food handling practices with milk and dairy products had been reported in the foodborne diseases in other countries (De Buyser *et al.,* 2001; Reyes *et al.,* 2007). An adequate control of storage temperature all along in the food chain is one of the most important points to guarantee the food safety in dairy products. According to several authors (Soriano *et al.,* 2001c, 2002b; Kassem *et al.,* 2002; Cenci-Goga *et al.,* 2005; Lievaart *et al.,* 2005), monitoring programs, including HACCP system, should be more extensive with particular attention to this food preparation. Nowadays, our group is carrying out the application of prerequisite programs and HACCP plan in these restaurants to guarantee the food safety in the milk and dairy products among other foods.

3.3.6. Meat and meat products

Our study reflected that from 9 to 20% of the studied lots of poultry, pig and beef samples exceeded the European and Spanish maximum level of mesophilic aerobic counts, while the same samples and the same range percentage-wise exceeded these

legislated levels for Enterobacteriaceae (Table 22). The others groups of meat analyzed had acceptable and marginal values for these microorganisms. Pedroso *et al.* (1999) detected in served meat products, including meat balls and kibbe, from a hospital kitchen, values for mesophilic aerobic counts of 3.4 and 3.8 $\log_{10}$ CFU g-1, respectively. Soriano *et al.* (2000b) observed a range from < 1.00 to 6.04 $\log_{10}$ CFU g-1 and < 3 to > 240 MPN g-1 for mesophilic aerobic counts and total coliforms, respectively, in meat product samples served in University restaurants. Arranz Santamaría *et al.* (1995) obtained values of 7.5 $\log_{10}$ CFU g-1 from meat products in bars and they suggested that due to incorrect processing and handling practices that this viewpoint is similar to that of other authors. Soriano *et al.* (2002a) demonstrated the efficiency of the HACCP system, together with inspection and personnel training, in the reduction of aerobic plate count, from 1.8 to 5.3 and from < 1.0 to 3.0 $\log_{10}$ CFU g-1, before and after implementation of the HACCP system respectively. This decrease in the levels of microbiological contamination was observed for pork loin samples in University restaurants.

For *E. coli*, 1 and 1.25% of studied samples had levels higher than legislated values from pig and poultry, respectively (Table 23). According to Moore *et al.* (2001) an improperly cleaned surface, if in contact with food can lead to cross contamination and contribute to the microbial load of a product, which might result in a decreased of its shelf life. The same research group recorded, in the UK, that cross contamination is an important

contributory factor in 39% of general food-borne disease outbreaks. De Wit and Rombouts (1992) suggested that *E. coli* is normally absent from hands and the presence of *E. coli* is thought to give a better indication of faecal contamination (enteric pathogens in particular) than the entire group of Enterobacteriaceae.

Table 22. Microbial quality, according to the European and Spanish legislations, for total aerobic mesophilic bacteria and Enterobacteriaceae in meat dishes collected from studied foodservice establishments.

	Microbial quality (%)					
	Total aerobic mesophilic bacteria			Enterobacteriaceae		
Source (no. of lots)	Acceptable	Marginal	Unacceptable	Acceptable	Marginal	Unacceptable
Poultry (n=80)	75	14	11	90	1	9
Pig (n=210)	82	9	9	85	2	13
Beef (n=91)	70	10	20	80	0	20
Others (n=4)	75	25	0	100	0	0

On the other hand, 0.5, 1 and 2.5% of the lots of pig, beef and poultry, respectively, exceeded legislated levels for *S. aureus* (Table 23). Ubach *et al.* (1988) and Soriano *et al.* (2000b) isolated this microorganism, in 2 and 4.8% of "ready-to-eat" meats. Soriano *et al.* (2002a) detected it in 17.9 and 2% of analyzed samples, before and after implementation of the HACCP system, respectively, in

pork loin samples. The presence of *S. aureus* from cooked foods have, according to Soriano *et al.* (2002f), a human origin and are introduced in food post heat treatment. This microorganism is usual in nose, throat, hands and nail samples of food handling personnel (Hattaka *et al.,* 2000). In fact, Mossel and van Netten (1991) indicated the presence of human carriers in kitchens as a useful indicator of cross-contamination.

In aircraft, Hatakka (2000) observed that some of the hot meals served exceeded the microbiological standards accepted by the Association of European Airlines (AEA, 1996) for *E. coli* (8.2%), *S. aureus* (0.6%) and *Salmonella* spp. (0.3%); while, in 9.2% of hot meal samples, total counts were higher than 6.0 $\log_{10}$ CFU g-1, which is the AEA limit for food items that have been handled after heat treatment.

Fortunately, in our study, neither *Salmonella* spp. nor *L. monocytogenes* were detected from any of the samples being similar results to our previous studies (Soriano *et al.,* 2000b, 2002a). *Salmonella* spp. was detected in 0.3 and 1% of hot meals by Roberts *et al.* (1989) and Lambiri *et al.* (1995), respectively. Cabedo *et al.* (2008) isolated *L. monocytogenes* in 11.1% of the pork luncheon meat samples from retail stores and food industry. Cardinale *et al.* (2005) detected *Salmonella* spp. in 20.1% of the 148 street-restaurants studied and in 10.1% samples of poultry dishes concluding that an increasing risk of *Salmonella* contamination is due to not peeling and not cleaning vegetables and other ingredients during meal preparation; dirty clothing for

restaurant employees, reheating previously cooked foods, and no kitchen and utensils disinfection are also associated with it. Furthermore, they observed that adequate cooking procedures decreased the risk of *Salmonella* contamination.

Table 23. Pathogenic microorganisms in meat dishes collected from studied foodservice establishments.

	POULTRY (n=80)	PIG (n=210)	BEEF (n=91)	OTHERS (n=4)
Staphylococcus aureus	2.5 %	0.5 %	1 %	n.d.
Escherichia coli	1.25 %	1 %	n.d.	n.d.
Salmonella spp.	n.d.	n.d.	n.d.	n.d.
Listeria monocytogenes	n.d.	n.d.	n.d.	n.d.

According to our study, Hartung (1993) demonstrated much lower levels of *Salmonella* Enteritidis in pig dishes in comparison with poultry dishes. Chudasama *et al.* (1991) did not find *L. monocytogenes* in any meat dishes in cook-chill food prepared by the Catering Department of the Royal Free Hospital in the UK, but in 5% of food samples, aerobic viable counts were detected with values greater than 5.0 $\log_{10}$ CFU g-1 while these levels were not found in meat dishes.

3.3.7. Fish and fish products

For prepared fish and cephalopods dishes, our analysis showed that from 14 to 30% of the studied lots exceeded the

European and Spanish maximum levels of mesophilic aerobic counts, while from 10 to 40% of these samples exceeded these legislated levels for Enterobacteriaceae (Table 24).

Table 24. Microbial quality, according to the European and Spanish legislations, for total aerobic mesophilic bacteria, Enterobacteriaceae in fish samples collected from studied foodservice establishments.

		Microbial quality (%)					
		Total aerobic mesophilic bacteria			Enterobacteriaceae		
Source (no. of lots)		Acceptable	Marginal	Unacceptable	Acceptable	Marginal	Unacceptable
Fresh water fish	Whitefish (n=20)	65	5	30	90	0	10
	Oily fish (n=21)	67	5	28	81	0	19
Salt water fish	Whitefish (n=67)	49	22	29	57	3	40
	Oily fish (n=63)	65	21	14	72	9	19
Cephalopods (n=56)		70	10	20	80	0	20

Neither *E. coli*, *S. aureus*, *Salmonella* spp. nor *L. monocytogenes* were detected in any studied samples from restaurants. In Spain, as in Australia (Dalton *et al.,* 2004), fish is one of the most common vehicles of foodborne outbreaks, but the bibliography about microbial analysis in fish dishes in restaurants is scarce. Nichols *et al.* (2000) detected that ice used to cool shellfish from retail and catering premises in the UK had a lower microbiological quality in comparison with ice samples used to

cool ready-to-eat fish. Schalch *et al.* (1999) detected *Clostridium perfringens* from fish pastes in Germany. Franco Monsreal *et al.* (1991) and Franco-Monsreal and Flores-Abuxapqui (1989) isolated *Vibrio parahaemolyticus* in seafood dishes from Panamanian restaurants and seafood handlers and seafood from restaurants in Mexico. Even if tuna salad is an unusual vehicle associated with foodborne outbreaks, this food caused an infection of *Campylobacter jejuni* (O:33) in a summer camp in the USA (Roels *et al.* 1998). Whereas *Salmonella* Typhimurium*, Salmonella* Montevideo and *Salmonella* Paratyphi were detected from fish and seafood dishes in Australia (OzFoodNet Working Group, 2004, 2006), and in fish-and-chip shops in the United Kingdom (Francis *et al.,* 1989), both infections were caused by salmon provided by the same catering firm in the UK.

On the other hand, no *Listeria* species were isolated from any type of fish dish (salmon, hake and sole) served in University restaurants (Soriano *et al.,* 2001a). *S. aureus* was detected in 2, 1 and 1 out of 28, 29 and 14 from codfish coquettes, grouper and salmon, respectively, from Spanish cafeterias being one of them proved to be enterotoxigenic, producing staphylococcal enterotoxin type A (Soriano *et al.,* 2002d). A seven percent of analyzed samples, including fish and shellfish, from restaurants and shops in the United Kingdom, were responsible, between 1969 and 1990, for 359 cases of staphylococcal food poisoning (Wieneke *et al.,* 1993).

3.3.8. Eggs and egg products

Table 25 shows microbial quality of egg and egg products in studied restaurants. No samples containing raw or undercooked eggs have been collected. All samples analyzed have received cooking procedures and treatments presumed sufficient to eliminate microorganisms. Five, sixteen and thirty-three percent of the Spanish potato, vegetable omelettes and hardboiled eggs, respectively, exceeded the European and Spanish maximum level of aerobic colony counts, while 11, 17 and 60% of these samples (vegetable, Spanish potato omelettes and hardboiled eggs, respectively) exceeded these legislated levels for Enterobacteriaceae (Table 25).

The other egg groups analyzed had acceptable and marginal values for these microorganisms. Soriano *et al.* (2001b) detected, in Spanish potato omelette, from University restaurants, aerobic colony counts from < 1.0 to 2.9 $\log_{10}$ CFU g^{-1} and total coliforms from < 3 to 43 most probable number (MPN) g^{-1}.

The highest value of unacceptable microbial quality was obtained with hardboiled eggs (Table 25) due, probably, to the fact these products are intensively handled after heat treatment and it can be classified as high-risk items. This product is usually used as an accompaniment to other meals and the procedure carried out is the following; to boil then turn off the heat and wait for 15 minutes, (a timer works well), to peal them while they are still warm, to cut and to serve in the dish, being the extraction of

peel by hand the critical control point above all with the wrong hand cleaner (Hernández *et al.,* 2003).

Table 25. Microbial quality, according to the European and Spanish legislations, for total aerobic mesophilic bacteria and Enterobacteriaceae in eggs and egg products collected from studied foodservice establishments.

Source (no. of lots)	Microbial quality (%)					
	Total aerobic mesophilic bacteria			Enterobacteriaceae		
	Acceptable	Marginal	Unacceptable	Acceptable	Marginal	Unacceptable
Spanish omelette (n=225)	93	2	5	90	3	17
Meat omelette (n=10)	100	0	0	100	0	0
Vegetable omelette (n=45)	84	0	16	89	0	11
Fried egg (n=5)	100	0	0	100	0	0
Hardboiled egg (n=15)	67	0	33	40	0	60

Fortunately, in our study, neither *Salmonella* spp., *E. coli*, *S. aureus* nor *L. monocytogenes* were detected from any of the samples collected at these establishments. In previous studies, *E. coli* was detected in 2% of Spanish potato omelette from University restaurants (Soriano *et al.,* 2001b) and *S. aureus* was detected in 3.5 (Soriano *et al.,* 2000b) and 2% (Soriano *et al.,* 2001b) of Spanish potato omelette in University restaurants. Values higher than previous cited studies were obtained by other authors (Arranz Santamaría *et al.,* 1995; Ferrer *et al.,* 1992). The presence of *S. aureus* indicates improper handling and possible

cross-contamination. According to data provided by the American Society for Microbiology (1996), restaurateurs do not wash their hands as often as they think they do. In fact, Soriano et al. (2002) reflected that after implementation of the HACCP system, together to inspection and personnel training, in University restaurants, *S. aureus* was not detected in any studied samples.

L. monocytogenes was detected in 3.1% of meals from restaurants and delicatessen shops in the city of Barcelona (Spain) (De Simón and Ferrer, 1998). Literature reflected that *Salmonella* spp. is the most common microorganism in egg and egg products in restaurants and the catering industry. Gonzalez Hevia and Mendoza (1995) found one strain of *Salmonella* enterica in human stools, another in a hen's egg, and the third in both stools and another egg pointing to large Spanish omelettes to be the contaminated food source in a summer camp. A case report of a miscarriage, at 16 weeks of gestation due to infection and transplacental passage of *Salmonella* group C, is reflected in the literature (Coughlin *et al.,* 2003) indicated, for this woman, the consumption, on holiday in Turkey, of an omelette that was undercooked. The largest outbreak of salmonellosis in Catalonia (Spain) to date and one of the largest reported worldwide, with 1435 cases and 117 hospitalizations, happened due to the consumption of a hard pastry with vanilla cream which was made with pasteurized liquid egg, but the dough of the coca was made with fresh egg (Camps *et al.,* 2005). In fact, in this case, it was also observed that the vanilla filling was cooled using the same work

surface employed to make the dough and this fact suggests a high risk of cross-contamination. Furthermore, the coca may have stayed at temperatures sufficient to allow bacterial multiplication for long enough to account for the mass infection. Spainsh foodborne infections by *Salmonella*, in the study of Arnedo *et al.* (1998), were caused by the consumption of food prepared with eggs, including fried or boiled eggs, omelette, soufflé and home-made russian salad. In a previous study, Perales and Audicana (1989) found *Salmonella* in 5 out of 372 eggs associated with foodborne disease with one strain isolated from the inside of the egg. Tansel *et al.* (2003) considered the omelette to be the source of a microbiologically outbreak in one squadron of a military battalion located in Edirne (Turkey), based on the epidemiological proofs.

3.4. Conclusions

In percentage, attending to mesophilic aerobic counts, vegetables are the most contaminated products, mainly broccoli (50%) and eggplant (40%) while egg- (hardboiled egg; 33%), fish- (whitefish from fresh water; 30%) and cereals-based (rice; 27%) dishes are the following products with high percentage of unacceptable microbial quality. Furthermore, attending to Enterobacteriaceae microbial quality, orange juice samples obtained from a stainless steel jug (81%) are the most contaminated, followed by vegetable- (broccoli; 70%) and egg-based (hardboiled egg; 60%) dishes.

On the other hand, *S. aureus* was found in vegetable- (lettuce, French beans and potato in a range from 1.2 to 8%), meat-based (pig, beef and poultry in a range from 0.5 to 2.5%) dishes and orange juice (1% of the analyzed lots). *E. coli* was detected in vegetable- (lettuce; 6.6%) and meat-based (pig and poultry in a range from 1 to 1.25%) dishes. *Salmonella* spp. was positive in vegetable-based (lettuce; 0.7%) dishes and orange juice (1%) sampled from a metal jug. *L. monocytogenes* was not detected in any studied samples. The presence of some studied microorganisms has shown that several handling practices require more attention, as is the incidence of *S. aureus* which indicates cross-contamination. For this reason, our results emphasize the importance of strict hygiene during handling practices in order to avoid contamination of the food product.

4. BIOLOGICAL-TOXICOLOGICAL ASPECTS OF *E. coli* HEAT LABILE TOXIN

4.1. Introduction

E. coli toxins can cause serious food poisoning in humans due to the ingestion of food or water contaminated with Enterotoxigenic *E. coli* (ETEC). Contamination of water with human sewage as well as infected food handlers may lead to contamination of foods. An infective dose of 10^6-10^{10} CFU/g. ETEC causes intestinal disease, traveler's diarrhea, which affects populations worldwide. Food poisoning caused by *E. coli* is usually caused by eating unwashed vegetables or undercooked meat. ETEC produces at least two types of enterotoxins: low molecular weight, heat-stable enterotoxin (ST), and high molecular weight, heat-labile enterotoxin (LT), which is the major virulent factor of ETEC. The severe losses of water and electrolytes which occur in *E. coli* infection appear to be caused by this toxin.

This dissertation is focused on Heat-Labile Enterotoxin, B subunit (LTB) structural characterization by using spectrometric and mass spectrometry methodologies (LC-ESI MS, MALDI-TOF MS). The aim of the research was also to determine the most efficient culture medium for *E. coli* growth and enterotoxin production with the objective to quantify by High-Pressure Liquid Chromatography with UV Detector (HPLC-UV) the amounts of LTB produced. *E. coli* strains from food samples were isolated and grown to determine if they were capable to produce LT.

4.2. Characterization of Heat-Labile toxin-subunit B from *E. coli*

4.2.1. Materials and Methods

4.2.1.1 Reagents

All chemicals were of the highest purity commercially available and were used without further purification. HPLC grade H_2O, CH_3CN, MeOH, NH_4HCO_3, and analytical-grade formic acid (FA) were purchased from Carlo Erba (Milan, Italy). Sinapinic acid (SA), α-cyano-4-hydroxycinnamic acid (CHCA), iodoacetamide, dithiothreitol (DTT), Ethylenediaminetetraacetic acid (EDTA), guanidine, trifluoroacetic acid (TFA) and tris-HCl were obtained from Sigma Aldrich (St. Louis, MO, USA). Modified trypsin sequencing grade was from Promega (Madison, WI, USA). Centriprep® cartridges having a 3KDa cut-off membrane and ZipTip™ were obtained from Millipore (Bedford, MA, USA). Bakerbond SPE™ C18 500 mg/3 ml was purchased from J. T. Baker.

Heat-Labile Enterotoxin, B subunit from *Escherichia coli* recombinant, expressed in *Pichia pastoris* was purchased from Sigma (Sigma Chemicals, St. Louis, USA). The enterotoxin was dissolved in water to prepare a 1mg/mL stock solution, which was maintained in frozen form until diluted.

4.2.1.2. High-Performance Liquid Chromatography/Electrospray Ionization-Mass Spectrometry (HPLC/ESI-MS)

LC ESI-MS was carried on a single quadrupole instrument (HP1100-MSD, Agilent Technologies, Santa Clara, CA, USA) using

C18 columns (Vydac, Hesperia, CA, USA; 2.1 × 250 mm). The eluents were 0.1% (v/v) TFA in HPLC-grade water (solvent A) and 0.1% (v/v) TFA in acetonitrile (solvent B). LTB was separated at a constant flow-rate of 0.2 ml/min, with a gradient of solvent A in the following proportions (v/v): started at 95% A for 15 min and with a linear gradient of 95-40% A in 75 min, then changed to 100% A in five minutes and in the next 10 min arrived at the initial conditions. The LTB protein stock solution was diluted in solvent A at a final concentration of 0.5 µg/µl.

4.2.1.3. MALDI-TOF MS

MALDI-TOF-MS experiments were carried out on a Voyager DEPRO mass spectrometer (PerSeptive Biosystems, Framingham, MA) equipped with delay extraction technology and N_2 laser at 337 nm. Mass spectra were acquired both in positive linear or in reflector mode and 10mg/mL SA and CHCA both dissolved in 50% ACN/0.1% TFA, were used as matrices for the analysis of proteins and peptides, respectively. MALDI-TOF analysis of intact protein was obtained in linear positive ion mode over the m/z range 8,000-14,400 and was averaged from about 150 laser shots.

For the analysis of the whole protein, 0.5 µl of LTB stock solution (1 g/l solution in water) were loaded on the stainless steel target together with 0.5 µl of SA.

The mixtures of LTB tryptic peptides were subjected to a desalting/concentration step with Zip-Tip C18 microcolumns prior to analysis by MALDI-TOF. Spectra were obtained in reflectron

positive ion mode over an *m*/*z* range 400–7000 and were averaged from about 250 laser shots. External calibration was performed by acquiring separate spectra of a mixture of standard peptides (Perseptive Biosystems). Identification of the protein fragments was carried out with MASCOT software from Matrix Science (http://www.matrixscience.com).

4.2.1.4. LTB in solution trypsin digestion

LTB protein folding can protect the amino acid chain from trypsin enzymatic cleavage. A preliminary step of denaturation, reduction and carboxymetilation of cysteine is necessary for an efficient LTB hydrolysis by trypsin.

LTB, 50 µg, was denatured at room temperature, in a guanidine buffer (6 M guanidine-HCl in 0.5 M Tris/Tris-HCl with EDTA 1 mM) at pH 8. Disulfide bridges were reduced by the addition of 10 mmol L^{-1} DTT for 120 min at 56 °C. SH groups were then alkylated with 55 mmol L−1 iodoacetamide in the above guanidine buffer, and maintained for 30 min in the dark. With the aim to remove reagents, the alkylated LTB was ultra-filtered on Centriprep® cartridges having a 3-KDa cut-off membrane, centrifuged at 14,000 g for 25 min and washed with NH_4HCO_3 50 mM pH 7.4. The retentate solution containing the purified and alkylated LTB, was digested by adition of trypsin at 1:50 (w/w) and was incubated overnight (approximately 14 h), at 37°C. The digestion was stopped by freezing at -20 ºC.

4.2.2. Results and discussion

Concerning the sequence information available in the literature, according to information supplied by the manufacturer, the LTB toxin purchased was derived from recombinant *Escherichia coli*, expressed in *Pichia pastoris* but no details about amino acid sequence neither molecular weight was provided, nor was it contained in any database because the amino acid sequence can change depending of the origin of the strain that produces the toxin.

LC/ESI MS analysis allowed the acquisition of the molecular weight of the recombinant LTB. The deconvoluted mass spectrum produces an average molecular mass of 12003±0.9 Da for the B chain studied (Figure 2B).

MALDI-TOF-MS analysis of the intact protein showed the presence of B chain of the heat labile enterotoxin with an MH+ value of 11999.38. The average molecular mass of the B chain allows a primary identification of LTB toxin (Figure 3).

The molecular weight measured by MALDI-TOF MS is comparable to that determined by LC/ESI-MS.

Moreover, the MALDI TOF mass spectrum acquired after the reducing and alkylating treatment, showed a mass increase of 114 Da suggesting the presence of one disulfide bond. Alkylation with IAA increases the mass of a peptide by 57.02 for each cysteine present (Park *et al.,* 2009). Reduction and alkylation of cysteine residues using DTT and iodoacetamide (IAA), respectively, minimizes the appearance of unpredictable mass signals from

disulfide bond formation and side-chain modification and improve detection of cysteine-containing peptides. Figure 3 shows the MALDI TOF mass spectra of the protein before (panel a) and after (panel b) treatment with IAA.

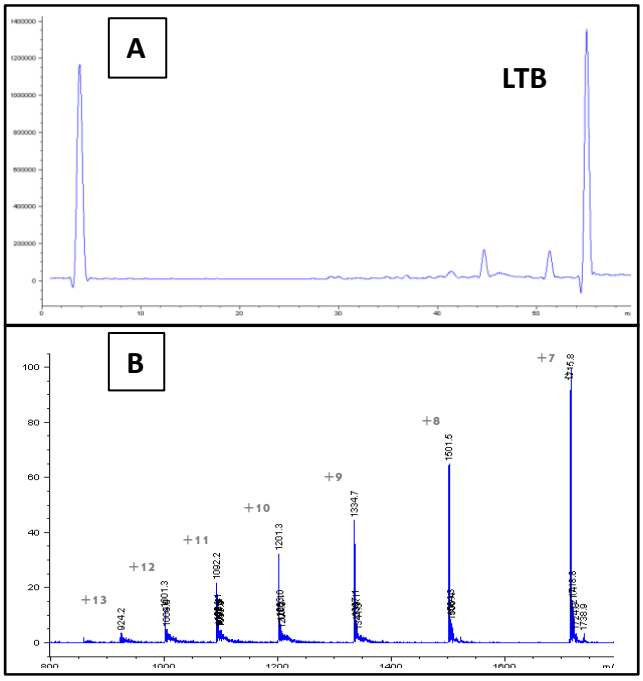

Figure 2. LC/ESI MS total ion chromatogram (A) of LTB protein and mass spectrum of *E. coli* toxin with ion abundance profile (B).

LC-ESI and MALDI-TOF MS analysis of the LTB standard showed a slightly higher molecular mass of the protein than masses previously reported (Yamamoto and Yokota, 1983). The aminoacid sequence of LTB toxin with different molecular masses has been described by several authors (Dallas and Falkow, 1980; Yamamoto and Yokota, 1983). The known natural variability of the aminnoacid sequences of LT toxins expressed by ETEC strains has been mainly restricted to the differences detected between LTs

produced by human (LTh) and pig (LTp) derived strains. Initial evidence based on the antigenicities and electrophoretic mobilities of LTh and LTp indicated that the toxins differ in their primary amino acid sequences (Honda *et al.,* 1981b; Tsuji *et al.,* 1982). Sequencing of the operons encoding LTh and LTp revealed differences in the primary sequences of the toxins, which share over 95% identity along the complete amino acid sequence (Vinal *et al.,* 1987).

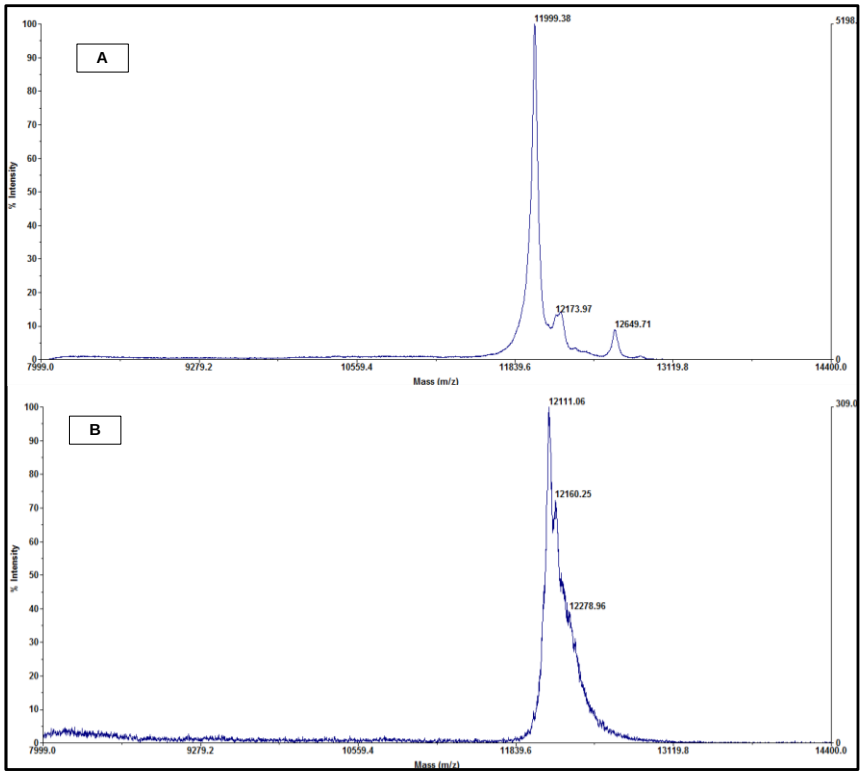

Figure 3. (A) MALDI-TOF MS analysis of LTB intact protein (B) MALDI-TOF results for LTB protein with disulfide bridges reduced by DTT and carboxymethylated with iodoacetamide.

Our results are similar to that of Van Baar *et al.* (1999), who studied cholera toxin, which is very similar to LTB in both structure and function. They studied two different batches of the toxin, purchased from different producers and found that were not in good order. This group concluded that in the stage of isolation or purification of the toxins the chains are apparently susceptible to damage and can suffer any sequence deviation. Takao *et al.* (1983) also worked with *E. coli* toxins and they showed the variability in the amino acid sequence of *E. coli* toxins when attempted to synthesize ST with the amino acid sequence reported in a previous work (Chan and Giannella, 1981) and found that the synthetic peptide had a different retention time on HPLC from that of the native toxin isolated from a strain. This finding suggested that the purified native toxin used had a different amino acid sequence from that proposed in a previous study (Chan and Giannella, 1981), although it had the same amino acid composition as that of their proposed sequence.

A more accurate confirmation of the sequence of the toxin can be obtained from enzymatic digestion with a verification of the digest fragment identity. In our study, the LTB subunit was reduced, carboxymethylated and subjected to a tryptic digestion. The digest mixture was analyzed by MALDI-TOF MS and results are shown in Figure 4.

Protein analyzed was identified by MASCOT software as "Heat-labile enterotoxin B chain OS=*Escherichia coli* O78:H11 (strain H10407/ETEC)", which aminoacid sequence is shown at Figure 5.

Sixteen peptides (range 400-4500 Da) were identified as products of trypsin cleavage of the LTB standard with a sore of 149 and 87% coverage of the protein sequence (Table 26).

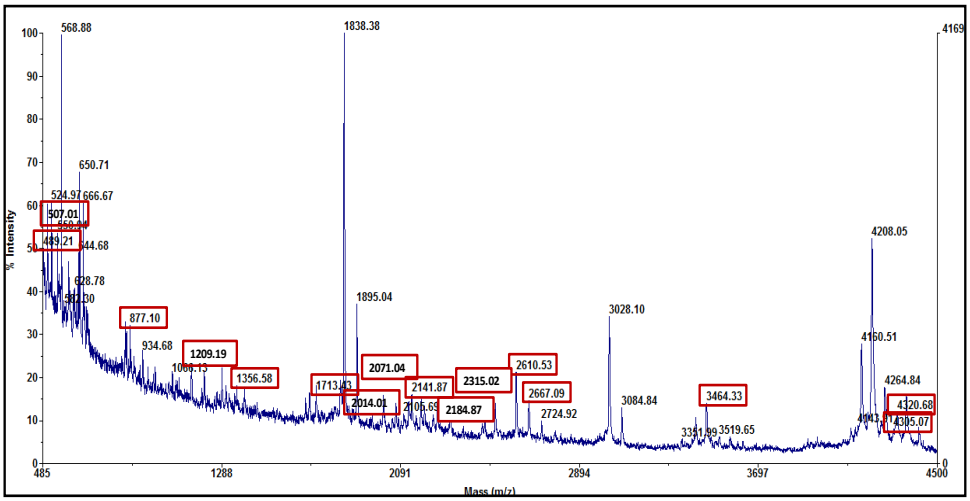

Figure 4. MALDI-TOF mass spectrum of trypsin digestion of LTB standard; identified LTB peptides in squares.

According to Godovac-Zimmermann *et al.* (2001), the ability to analyze increased numbers of peptides corresponding to higher sequence coverage is essential both to improve the reliability of protein identification and for more complete characterization of protein primary structure. However, any fragment corresponding to N-terminal side of the protein was identified. Furthermore, the three more intense peaks appearing in the mass spectra (*m/z* 1838.38, 3028.10 and 4208.05) cannot be assigned to peptide fragments of the digested protein.

Table 26. MALDI-TOF MS assignments of peptides after tryptic digestion from *E. coli* LTB.

Sequence range	$[M + H]^+$ calculated	$[M + H]^+$ observed	Peptide sequence
35-44	1208.60	1209.19	NTQIYTINDK
45-56	1355.69	1356.58	ILSYTESMAGKR
45-64	2313.99	2315.02	ILSYTESMAGKREMVIITFK
57-94	4319.99	4320.68	EMVIITFKSGATFQVEVPGSQHIDSQKKAIERMKDTLR
65-83	2013.97	2014.01	SGATFQVEVPGSQHIDSQK
65-84	2142.07	2142.01	SGATFQVEVPGSQHIDSQKK
65-88	2609.99	2610.53	SGATFQVEVPGSQHIDSQKKAIER
65-102	4305.24	4305.07	SGATFQVEVPGSQHIDSQKKAIERMKDTLRITYLTETK
84-90	876.01	877.10	KAIERMK
84-105	2665.99	2667.09	KAIERMKDTLRITYLTETKIDK
85-88	487.97	489.21	AIER
85-102	2183.57	2184.87	AIERMKDTLRITYLTETK
89-102	1711.91	1713.43	MKDTLRITYLTETK
89-105	2069.84	2071.04	MKDTLRITYLTETKIDK
91-94	506.27	507.01	DTLR
95-124	3465.16	3464.33	ITYLTETKIDKLC*VWNNKTPNSIAAISMEN

*Cysteines treated with iodoacetamide.

When these non-identified fragments were studied, it was noticed that all of them corresponded to N-terminus fragment with none, one or two tryptic missed cleavages, respectively (Figure 6). Furthermore, all these signals had a mass increase of 283 relative to previously identified LTB sequences. From these data the serine involved in modification can be indicated as serine 15 or 21 in the sequence.

This change may be caused by a modification during production or purification like suggested by Van Baar *et al.* (1999). According to Delta Mass Database (http://www.abrf.org/index.cfm/dm.home?AvgMass=all), mass

increase may be due to an O-GlcNAc-1-phosphorylation (of serine). In fact, during the past two decades, it has become clear that O-GlcNAc is one of the most abundant posttranslational modifications within the nucleocytoplasmic compartments of plants, animals, bacteria and viruses (Hart and Akimoto, 2009).

1	MNKVKCYVLF	TALLSSLCAY	GAPQSITELC	SEYR**NTQIYT**	**INDKILSYTE**
51	**SMAGKREMVI**	**ITFKSGATFQ**	**VEVPGSQHID**	**SQKKAIERMK**	**DTLRITYLTE**
101	**TKIDKLCVWN**	**NKTPNSIAAI**	**SMEN**		

Figure 5. Amino acid sequence of Heat labile enterotoxin B chain (LTB) *Escherichia coli* O78:H11 (strain H10407 / ETEC) with signal peptide.

O-GlcNAc modification is commonly found in bacteria toxins, for example, the α-toxin of the gangrene causing bacteria *Clostridium novyi* is an O-GlcNAc transferase that exerts its toxic effects by the addition of O-GlcNAc to proteins in the Rho subfamily (Selzer *et al.,* 1996). Thus, the disruption of normal O-GlcNAc-regulated pathways may be responsible for the pathology of some bacteria. Moreover, disruption of the gene for O-GlcNAc transferase demonstrates that O-GlcNAc modification is essential for life, even at the single cell level (Shafi *et al.,* 2000). Considering these changes, the protein sequence has been identified with 100% coverage.

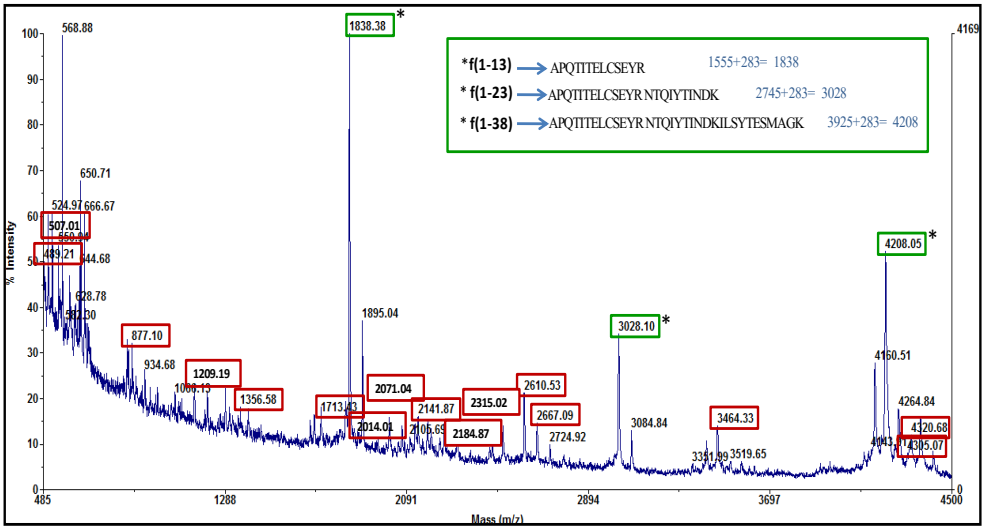

Figure 6. MALDI–TOF mass spectrum of LTB tryptic digestion. In the box the LTB N-terminal tryptic peptides are reported.

4.3. Effect of different nutrient media on production of heat labile enterotoxin by *E. coli*.

4.3.1. Material and methods

The strains used in this study were *E. coli* CECT 385 and DSM 10973. Both of them were purchased as toxigenic strains producers of heat labile enterotoxin. One non-toxigenic *E. coli* strain CECT 405 was used as a negative control for LT production.

Lyophilized *E. coli* strains were initially reconstituted in a nutrient broth and incubated at 37 ºC with constant shaking. Turbidity was read periodically until arrive at stationary phase and then 1 ml of each strain was plated onto a selective media for *E. coli,* (CHROMagar Microbiology, Paris, France). After incubation for 48 hours at 37 ºC, seven colonies from each plate were inoculated into 100 ml of different culture media in 250 ml flanged flasks.

4.3.1.1. Bacterial growth

Chopped Meat Broth, Tryptic Soy Broth, Luria-Bertani Broth and Brain Heart Infusion were each inoculated with each strain. In selected experiments attempts were made to identify which culture media has chemical factors that favor heat labile enterotoxin production by the *E. coli* strains tested, CECT 405, CECT 385 and DSM 10973. In all experiments microorganisms were cultured at 37°C with high aeration by constant shaking at approximately 200 rpm. To test bacterial growth, absorbance of all cultures was measured at different times (0, 4, 8, 24, 32, 48 and 72 hours).

4.3.1.2. Toxin production

Every 24 hours, 1 ml of each culture was tested for HLT production with the VET-RPLA toxin detection kit (Oxoid). The VET-RPLA test is designed for the detection of LT or CT in culture fluid. A positive result given in the test indicates that the organism produces the relevant enterotoxin. The procedure involves a suspension of heat labile enterotoxin antibody-coated latex. The LT enterotoxin has antigenic structures similar to those found on *Vibrio cholerae* enterotoxin. Antiserum taken from rabbits, immunized with CT, will therefore react with both CT and LT. Cultures were filtered with 0.22-µm hydrophilic regenerated cellulose filter membranes, and the filtrate was retained for an assay of the toxin content.

Cell-bound toxin was also determined in whole cell extracts. Bacterial cultures were centrifugated and pellet was separed from supernatant. Bacteria cells were suspended in 3 ml of phosphate-buffer saline (PBS) buffer (100 mM phosphate, 150 mM NaCl, pH 7.2) followed by sonic disruption with a Branson sonifier 450D according to Lasaro *et al.* (2007). Treatment with chloroform allows a simple, rapid, and quantitative release of periplasmic proteins. Therefore, 10µl of chloroform for each ml of culture tested were added to the bacterial pellet. Cell debris were removed by centrifugation at 4,000 rpm for 10 min at 4º C and supernatants assayed by VET-RPLA kit to test toxin content. The microtiter plates of the VET-RPLA kit were sealed with a plate sealer and shaken to mix the contents of the wells. Immediately after that, the

plates were incubated at room temperature on a vibration-free surface, and the agglutination reactions were read after 20 to 24 h by holding the plates against a dark background with indirect lighting.

4.3.1.3. HPLC-UV analysis

Positive VET-RPLA bacterial cultures were also tested by HPLC-UV to confirm and quantify the toxin produced. One ml of the culture filtrates was centrifuged at 13000 rpm for 15 minutes. Supernatants were lyophilized and denatured at room temperature in a guanidine buffer (6 M guanidine-HCl in 0.5 M Tris/Tris-HCl with EDTA 1 mM) at pH 8. Disulfide bridges were reduced by the addition of DTT for 120 min at 56 °C. Free cysteine residues were then carboxymethylated with an excess of iodoacetamide in the above guanidine buffer, and maintained for 30 min in the dark. The reactant solution was transferred to Centriprep® cartridges having a 3-KDa cut-off membrane a MWCO 3,000 filter, centrifuged at 14,000 g for 25 min and washed twice with 400 µl NH_4HCO_3 buffer.

HPLC-UV analysis was performed using a HP 1100 modular HPLC apparatus (Agilent, Palo Alto CA, USA). A Jupiter C18 reversed-phase analytical column (150 x 2mm, 3µm) Phenomenex column was used with a flow rate of 0.2 ml/min. The volume injected of standards and sample solutions was 20 µl. Solvent A consisted of 0.1% TFA in water, and solvent B was 0.1% TFA in ACN. HPLC conditions were set up using the gradient shown at

Table 27. LT-B was detected at 205nm. Enterotoxin identification was performed by comparing retention time and UV spectra of purified extracted samples to pure standards.

Table 27. Gradient program used for a 15 cm Jupiter column.

Time (min)	Solvent A (%)	Solvent B (%)
0	80	20
15	60	40
30	40	60
40	80	20

4.3.1.4. Application to *E. coli* isolated from food samples

Six strains of *E. coli* isolated from food samples collected at university restaurants (2 milk, 3 meat and 1 lettuce samples) were subcultured and tested by VET-RPLA and HPLC-UV for LT production.

4.3.2. Results and discussion

E. coli strains showed to be capable of grow in the four media cultures inoculated. Nevertheless, bacteria do not achieved the same growth levels in all media tested. In chopped meat broth strains CECT 405 and DSM 10973 reached stationary phase with high absorbance values, indicating optimal growth. However, strain CECT 385 did not reach the same concentration. In the other three culture media, all the strains achieved similar concentrations but only in TSB cultures reached stationary phase after 24 hours (Figure 7).

VET-RPLA test results indicated that only two culture media induced toxin production under the conditions tested. Tryptic soy broth showed positive results for the DSM 10973 strain; the chopped meat broth culture inoculated with the same strain was also positive for LT.

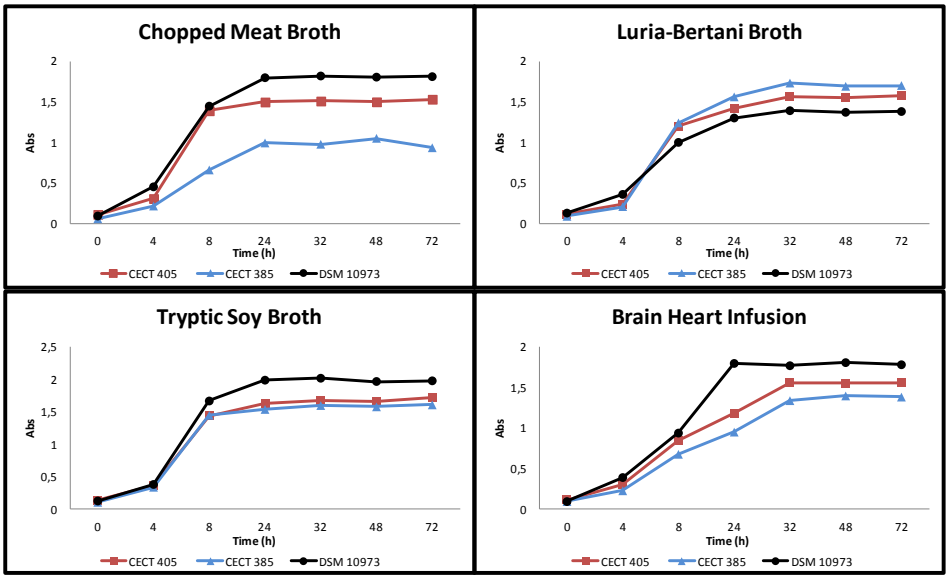

Figure 7. Efficiency of different culture media broth on the growth of *E. coli* strains.

Toxin production was only detected in cultures once stationary phase had been achieved (after 32 hours). The results obtained for the control strain (non toxigenic CECT 405) and CECT 385 were always negative. For the other two culture media, the tests were always negative, indicating that detectable amounts of LT were not produced when strains were grown and treated in this way (Table 28).

Similarly, the analysis of the bacterial pellets from all the media tested gave negative results, which could mean that cell-associated LT extraction did not work properly and enterotoxin was not extracted. However, according to the work of Lasaro *et al.* (2007) is most probably that undetectable levels of LT have been produced by the strains. This group compared different permeabilization treatments to release the toxin from the bacterial cells and found that maximal LT levels were detected in whole cells extracts submitted to the sonic treatment, they concluded that sonic disruption is the most efficient LT-releasing procedure. However, they also confirmed previous studies conclusions, that production and release of LT can vary among different ETEC strains. They determined that the production of LT among 26 LT producing ETEC strains ranged from a minimum of 49.8 ng/mL to more than 2,400 ng/mL (Lasaro *et al.,* 2006).

Several authors have found LT in supernatant extracts (Dorner, 1975; Clements and Filkelstein, 1979; Hegde *et al.,* 2009) but LT has been also isolated into whole cells lisate by Lasaro *et al.* (2007). Although they found mainly toxin in cell lisates, various levels of LT were also released in culture supernatant, even reaching 50% of the total synthesized toxin. During these experiments it was noted that both strains tested showed great variability for LT production. It is proposed that variability in an as-yet-unidentified nutrient in the culture media tested explains the variation in toxin amounts obtained.

Table 28. VET-RPLA results of different *E. coli* strains cultured in several broth.

Culture Media	Strains tested	VET-RPLA RESULTS							
		Hours							
		0	4	8	24	32	48	72	Pellet
Chopped Meat Broth	CECT 405	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	CECT 385	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	DSM 10973	n.d.	n.d.	n.d.	n.d.	+	+	+	n.d.
Luria-Bertani Broth	CECT 405	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	CECT 385	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	DSM 10973	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Tryptic Soy Broth	CECT 405	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	CECT 385	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	DSM 10973	n.d.	n.d.	n.d.	+	+	+	+	n.d.
Brain Heart Infusion	CECT 405	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	CECT 385	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	DSM 10973	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d. = not detected

Results obtained by VET-RPLA test were also confirmed by HPLC-UV technique. Under the chromatographic conditions used in this study, the calibration curve exhibited good linear regression (r^2=0.998) and the limits of detection (LOD) and quantification (LOQ) were 0.5 and 1ng, respectively. Both limits were calculated according to s/n=3 and s/n=10, respectively. Retention time for LTB was 33.1 min. The recovery assays of the B chain were carried out by adding the toxin standard to the bacteria culture inoculated with the non toxigenic strain tested (CECT 405). Recovery for toxin assayed was 83.9% with a standard deviation of 1.3%.

Tryptic soy broth and Chopped Meat Broth inoculated with the DSM 10973 strain were tested by HPLC-UV. For tryptic soy broth the amount of LT-B found was 49 ng/mL whereas for chopped meat broth only 36 ng/mL were quantified (Figure 8). In this study, we noticed that the amount of enterotoxin produced by strain DSM 10973 is quite variable and were unable to identify the source of the variability. Comparing our results with other works we can conclude that levels of enterotoxin production are in the range of levels found by other authors with different growing conditions and wild and reference strains (Lasaro *et al.,* 2006; Hegde *et al.,* 2009).

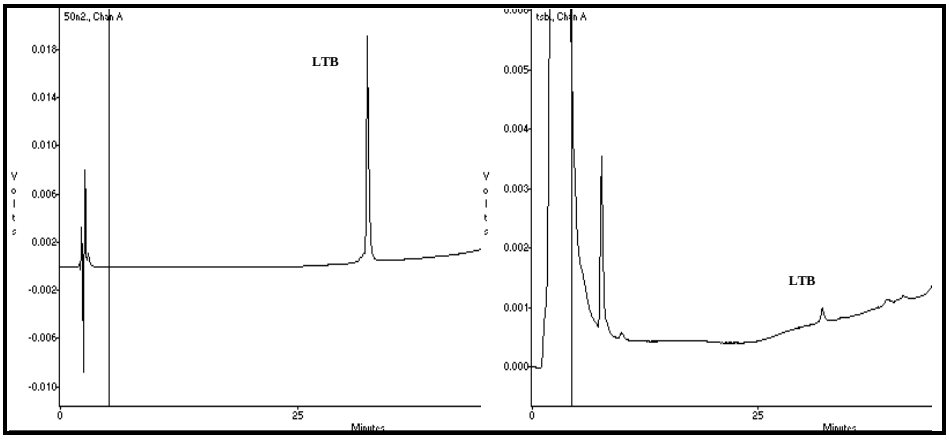

Figure 8. HPLC-UV chromatograms of a LTB standard and tryptic soy broth supernatant inoculated with DSM 10973.

Tryptic soy broth was selected as culture medium for the following tests because of its ability to favour bacteria growth and toxin production. *E. coli* strains isolated from food samples

collected at the university restaurants (2 milk, 3 meat and 1 lettuce samples) were tested for enterotoxin production.

Several cases of foodborne diseases caused by ETEC involving milk, meat and vegetables samples can be found in literature (Ochoa *et al.,* 2009; Chongsuvivatwong *et al.,* 2009; Ethelberg *et al.,* 2010). Nevertheless, none of the strains isolated and tested in our showed to be enterotoxigenic. Milk samples are frequently involved in intestinal diseases in infants in developing countries, where incidence of ETEC infection is highest in the first two years of life (Ochoa *et al.,* 2009; Taneja and Merson, 2003). However, ETEC outbreaks caused by contaminated food consumed in food establishments occur relatively rarely. Nevertheless, some outbreaks caused by ETEC have been reported recently. In 2010 in Dermark a series of outbreaks of gastroenteritis were reported to Danish authorities. All outbreaks occurred within groups of people (company employees, course attendees etc.) who had lunch delivered from catering companies. The food in each case included sandwiches or Danish-style open sandwiches. Comparison of ingredient lists identified lettuce as the only relevant common food item. Analysis for pathogenic bacteria (*Salmonella*, *Campylobacter, Shigella* and *Yersinia*) were negative, but examination for diarrhoeagenic *E. coli* revealed the presence of ETEC in 11 cases of 24 examined (Ethelberg *et al.,* 2010). Some cases of travelers' diarrhea in Thailand have been also studied and eating outside the hotel and eating meat were the main risk

factors. ETEC was found in 7 of 56 subjects studied (Chongsuvivatwong *et al.,* 2009).

4.4. Conclusions

It was shown that subunit B of *E. coli* heat labile enterotoxin can be primarily characterized by a molecular mass determination by MALDI-TOF MS and LC/ESI MS. The presence of one intramolecular disulfide bridge in the B-chain protein is readily verified by IAA reduction and MALDI-TOF MS.

Tryptic soy broth has shown to be the most efficient culture medium for *E. coli* growth and enterotoxin production, even so LT production by *E. coli* can vary among ETEC strains and depending on the culture conditions. No food sample from university restaurants was contaminated by LT producer strains of *E. coli*.

5. BIOLOGICAL-TOXICOLOGICAL ASPECTS OF STAPHYLOCOCCAL EXOPROTEINS

5.1. Introduction

Staphylococcus aureus is a human and animal pathogen that can produce numerous toxins, including pyrogenic toxins, like staphylococcal enterotoxins and toxic shock syndrome toxin 1. TSST-1 causes toxic shock syndrome, a disease which symptoms were first described in children who presented high fever, headache, confusion, conjunctival hyperaemia, scarlatiniform rash, subcutaneous oedema, vomiting, diarrhoea, refractory hypotension, oliguria and acute renal failure. About 15-40 per cent of healthy humans are carriers of *S. aureus*. As the human nares and fingers are the important sources of *S. aureus*, human carriers can also contaminate food or work surfaces from the hands of food handlers if proper care is not taken when preparing and serving food (Polledo *et al.*, 1985). Therefore, enterotoxigenical strains may contribute to staphylococcal food poisoning, as any staphylococcal strain can be involved in food poisoning.

Measures for consumer protection at university restaurants, as well as food quality are important to assess the cleanliness and the incidence of TSST-1 on work surfaces.

Once microbiological quality of food served at restaurants has been evaluated, the next step is to analyze most common used work surfaces, just as workers´ hands for the presence of TSST-1 in restaurant kitchens.

The aim of this part of dissertation thesis is to evaluate, in university restaurants, the incidence of TSST-1 on food handlers and on work surfaces, the optimization and application in milk samples of methods for SEA detection by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS), quantitation of SEB by LC-DAD and simultaneous quantification of SEA and SEB by LC-MS. Furthermore, SET-RPLA and LC-ESI/MS techniques have been applied to determine the presence of several SEs produced by *S. aureus* strains isolated from university dishes.

5.2. Study of TSST-1 from *S. aureus* isolated in food handlers and university foodservice establishments.

5.2.1. Materials and methods

5.2.1.1. Samples and sampling

A total of 908 samples from surfaces were collected from 2008 to 2011 in different Spanish restaurants. Surfaces analyzed included worker's hands, stainless steel tables, dish towels, cutting boards, slicers, clean plates and kitchen knives. All samples from food service establishments were collected and analyzed the same day. According to the study design approved by the Committee on Ethical Research of the University of Valencia (Spain), a written consent was obtained from food handlers.

Surface sampling was done using a single swab method. Sterile cotton collection swabs moistened in sterile BPW were rolled several times over the surface of any item to be sampled.

Swabs were inoculated in 10 ml of BPW, vortexed and incubated at 37 ºC for 24 h. The homogenate in BPW was subcultured onto BP agar supplemented with egg-yolk emulsion and incubated at 37 ºC for 24-48 h.

5.2.1.2. Bacterial identification

The isolates were identified on the basis of cultural characteristics (typical black, convex colonies and with or without light halo), Gram stain reaction and the results of catalase and tube coagulase tests. Tube coagulase negative, catalase positive and gram-positive coccal isolates were identified further with biochemical test (API Staph identification system; bioMérieux SA, Marcy l'Etoile, France). Isolated and identified staphylococcal strains were inoculated into 10 ml of tryptone soya broth (Oxoid) for 24 h at 37 ºC with constant shaking. The cultures were filtered with 0.22 μm hydrophilic regenerated filter membranes (Phenex-RC, Phenomenex, Micron Analítica, Madrid, Spain) and supernatant was retained for an assay of the toxin content.

5.2.1.3. Toxin production

Culture filtrates were studied by TST-RPLA (Oxoid) as directed by the manufacturer. The TST-RPLA test is a kit designed for the detection of staphylococcal toxic shock syndrome toxin in culture filtrates by reversed passive latex agglutination. A positive result given in the test indicates that the organism produces the relevant toxin. The procedure involves a suspension of toxic shock

syndrome toxin antibody-coated latex. Polystyrene latex particles are sensitized with purified antiserum taken from rabbits immunized with purified TSST-1. These latex particles will agglutinate in the presence of TSST-1. A control reagent is provided which consists of latex sensitized with non-immune rabbit globulins. The microtiter plates of the TST-RPLA kit were sealed with a plate sealer and shaken to mix the contents of the wells. Immediately after that, the plates were incubated at room temperature on a vibration-free surface, and the agglutination reactions were read after 20 to 24 h by holding the plates against a dark background with indirect lighting.

5.2.2. Results and discussion

The analysis showed that 5.8% of total studied samples were contaminated by *S. aureus* being mainly in dish towels (10.1%) followed by workers' hands (8.4%), cutting boards (6.5%), stainless steel tables (5.8%) and slicers (3.4%) (Table 29). Neither clean plates nor kitchen knives were positive for *S. aureus*.

Some studies reflected the presence of TSST-1 in *S. aureus* isolated from food samples around the world. Oh *et al.* (2007) detected TSST-1, in 13.5% strains of the toxigenic isolates from food in Korea. Rapini *et al.* (2005) found that 4.4% of the *S. aureus* isolated from goat's cheese handlers, produced TSST-1. Dallal *et al.* (2010) detected it in twelve strains from a total of 100 *S. aureus* strains isolated from 1047 food samples. TSST-1 was detected in *S. aureus* isolated in milk from cows with clinical and subclinical

mastitis, and in farm bulk tank milk (Takeuchi *et al.,* 1998). In three strains out of 40 Libyan *S. aureus* clinical strains was detected tst gene encoding TSST-1 but in none of the strains isolated from food (El-Ghodban *et al.,* 2006). Tsen *et al.* (1998), in Taiwan, employed a PCR assay, with the TSST-1- specific primers, to identify as tst-carrying strains only three (4.8%) of 62 strains of *S. aureus* isolated from clinical sources, but none of the food strains studied carried this gene. These studies demonstrate the high frequency *S. aureus* in foods served at restaurants.

Table 29. Incidence of *Staphylococcus aureus* and TSST-1 isolated from them in food handlers and foodservice establishments in Spain.

Source	***S. aureus* (%)**	**TSST (%)**
Workers' hands (n=227)	19 (8.4)	1 (5.3)
Stainless steel tables (n=138)	8 (5.8)	nd
Dish towels (n=138)	14 (10.1)	nd
Cutting boards (n=138)	9 (6.5)	nd
Slicers (n=89)	3 (3.4)	nd
Clean plates (n=89)	0	nd
Kitchen knives (n=89)	0	nd
Total (n=908)	**53 (5.8)**	**1 (0.1)**

nd: not detected

Bacteria present in foods may be destroyed by some cooking processes, as heat treatment but even so, toxins produced by these bacteria can arrive at final consumers. For this reason, surfaces and food handlers hygiene has mainly importance to prevent foodborne outbreaks. Several studies indicated that various bacteria, including *E. coli, S. aureus* and *Salmonella* spp., survive on hands, sponges/cloths, utensils and currency for hours or days after initial contact with the microorganisms (Scott and Bloomfield, 1990; Jiang and Doyle, 1999). The extent of bacterial survival and cross-contamination between hands and foods or various kitchen surfaces have been quantified by some authors (Chen *et al.*, 2001b; Montville *et al.*, 2001). The retention of bacteria on food contact surfaces increases the risk of cross-contamination of these microorganisms to food. Cross-contamination occurs when safe to eat food comes into contact with pathogenic bacteria, chemicals or unwanted items making the food unsafe to eat. Many pathogens are passed between people through improper hand washing when handling food. If contaminated hands with TSST-1 producer strains touch foods cross-contamination can occurs. Hand washing is the best way to prevent the spread of disease. Hands must also be washed before starting work, before handling any food, whenever they are visibly dirty or after finishing one job and before beginning another job. In some of studied restaurants the use of gloves was a widespread practice. However, using gloves does not replace hand washing, is only a tool and must be used properly to ensure food safety.

The current legal framework, either national or European level, does not mention the use of gloves neither the material should be made to preserve the hygiene of foodstuffs. The wearing of gloves to handle food is not required by law in Spain but it was a common practice until few years ago, when Spanish Agency for Food Safety and Nutrition made a recommendation to limit the use of latex gloves in food practice, relegating its use to cases where strictly necessary for the job characteristics or worker, such as having sores on their hands (AESAN, 2008). The use of gloves in food practice unconsciously may lead to poorer hygiene, can lead to less attention to hand washing, it is necessary always after handling foods that can transmit diseases, such as materials raw crude, and poor hygiene can cause cross-contamination of food.

Other works showed that good handling practices are important to prevent poisoning on the final consumers. According to our results, Fueyo *et al.* (2005a) found a high level of samples contaminated by *S. aureus*, his group isolated 269 *S. aureus* from nasal carriers and manually handled foods in Spain, being ten isolates producing only toxic shock syndrome toxin (TSST-1) and ten isolates producing enterotoxins and TSST-1. Udo *et al.* (2009) detected the presence of the TSST-1 in thirteen isolates, including two *S. aureus*, ten coagulase-negative staphylococci (5.6%) from hands and one nasal *S. hominis* from restaurant workers in Kuwait. In milk handlers from an Argentinean factory of dairy products, Puig de Centorbi *et al.* (1990) detected TSST-1 in three out of eight *S. aureus* strains isolated.

In our study only 0.1% of all the surfaces studied was found contaminated by TSST *S. aureus* producer. The toxigenic strain was isolated from an employee's hand who was working in one of the studied restaurants (Table 1).

Adesiyun *et al.* (1992) concluded that TSST-1 producing strains of *S. aureus* are widespread in humans, animals and foods in Nigeria. However, in Spain this is the first time that TSST-1 producing *S. aureus* is detected in a worker hand from foodservice establishment.

5.3 Analysis of staphylococcal enterotoxin A in milk by MALDI-TOF MS

5.3.1. Materials and methods

5.3.1.1. Samples and sampling

All organic solvents used in this study were of "proanalysis" (p.a.) quality. Coomassie brilliant blue R-250 (CBB) was supplied by Bio-Rad (CA, USA). IAA, DTT, trypsin proteomics grade, and Staphylococcal enterotoxin A were purchased from Sigma (St Louis, MO, USA). The enterotoxin was dissolved in water to prepare a 1mg/mL stock solution, which was maintained in frozen form until diluted (serially) to make additional standard solutions of 100, 10, and 1 ng/μL, according to Callahan *et al.* (2006). Care should be taken in the handling of SEA, using gloves and protection at all times. Surfaces and materials exposed to SEA should be treated with bleach to destroy residual toxin.

5.3.1.2. Extraction of staphylococcal enterotoxin A from milk.

A sample of skimmed cows' milk was spiked with different concentrations of enterotoxin. This milk (1 mL) was purified by the following steps. Milk protein precipitation was performed with 200 μL dichloromethane and 100 μL water containing 5% acetic acid at pH 4.5 before centrifugation for 15 min at 5,000 rpm (Chen *et al.,* 2004). Protein samples were diluted 1:2 in Laemmli sample buffer and the mixture was heated for 5 min at 95 °C, then put into ice for 30 s to cool to room temperature and applied to the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

5.3.1.3. SDS-PAGE separation

One-dimensional SDS-PAGE was performed with 10% polyacrylamide gels according to the method of Laemmli (1970) in a Bio-Rad Protean II electrophoresis system. Gels were stained overnight in 0.2% (w/v) CBB R250 45% (v/v) methanol 10% (v/v) acetic acid in water. The destaining solution was 40% (v/v) methanol 10% (v/v) acetic acid in water.

5.3.1.4. MALDI sample preparation

In-gel digestion with trypsin was performed according to standard procedures (Jenö *et al.,* 1995; Shevchenko *et al.,* 1996) with minor modifications. Coomassie stained protein bands were excised from the gel and washed three times for 10 min with water (LC grade; Merck, Darmstadt, Germany), equilibrated with 100 μL

50 mmol L^{-1} NH_4HCO_3 (pH 7.8), shrunk with acetonitrile, rehydrated with 100 μL 50 mmol L^{-1} NH_4HCO_3 (pH 7.8), and finally shrunk again with acetonitrile. The disulfide bonds were reduced with 10 mmol L^{-1} DTT for 30 min at 56°C and SH groups were subsequently alkylated with 55 mmol L−1 iodoacetamide, and maintained for 20 min in the dark. The supernatant was removed, and the gel was washed with 100 μL 100 mmol L−1 NH_4HCO_3 for 10 min then shrunk with acetonitrile. The gel pieces were reswollen in a digestion buffer containing 50 mmol L−1 NH_4HCO_3 (pH 7.8), and treated with 0.2 μg trypsin (Promega, Madison, WI, USA) at 37°C overnight.

Peptides were extracted by subsequent incubation with 0.5% trifluoroacetic acid (TFA)-acetonitrile (1:1, v/v) and the extract was evaporated to dryness. Finally, 0.1% TFA- 70% acetonitrile was added for 5 min then the solution was evaporated to dryness. The pellet was dissolved in 10 μL 0.1% TFA, and 0.5 μL was spotted on a MALDI target plate simultaneously with 0.5 μL of matrix solution. The matrix solution was prepared such that a saturated solution of CHCA dissolved in a mixture of acetonitrile (35% v/v) and aqueous 0.1% TFA (65% v/v) resulted. The internal standards used were Peptide Calibration Standard (Bruker Daltonics, Bruker) at 10 pmol μL^{-1}. Matrix and standards were mixed in equal volumes and then added to an equal volume of sample before 0.5 μL of each was spotted on to the AnchorChip 600/384 T F target plate (Bruker Daltonics) and allowed to dry.

5.3.1.5. MALDI-TOF mass spectrometry

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry was performed on a Reflex IV Bruker Daltonics instrument (Bruker–Franzen Analytic, Bremen, Germany). Identification of the protein fragments was carried out with MASCOT software from Matrix Science (http://www.matrixscience.com).

5.3.2. Results and discussion

Successful identification of protein profiles using MALDI relies on acceptable mass resolution and reproducibility of the mass spectra. The intraday and interday variation was assessed by performing repeated sample preparations and analysis during three consecutive days; this consisted of sample preparation, extraction, and analysis in triplicate. However, the quality of MALDI mass spectra depends on experimental conditions, for example sample pre-treatment and selection of matrices and samples.

In this work, one of the main problems was the high fat and protein content of the milk, which can interfere with the analysis. With the objective of resolving this drawback, the procedure for extraction of SEA from milk was based on a previous study in which Chen *et al.* (2004) replaced trifluoroacetic acid with acetic acid to fulfil the instrumental requirements facilitating analysis of the toxins. The use of dichloromethane not only dissolves milk fat but also causes the fat layer to sink below the casein precipitate layer. The desired proteins are thus free from interferences from

casein and fats. Clear SEA bands were obtained on the CBB stained gel. The range studied was between 150 ng and 750 ng. The same purification method was applied to an untreated sample of milk and no band around 27 kDa appeared.

The SEA band was excised from the gel, ground to small pieces, and treated with DTT and IAA to reduce and block possible disulfide linkages. After digestion with trypsin, the fragments were extracted and MALDI-TOF MS analysis was performed on the in-gel tryptic digests. One-dimensional gel analysis is less time-consuming, has the advantage of small amounts of proteins required, and yet yields reproducible results. Furthermore, 1D gel analysis seems to enable detection of some alkaline proteins as SEA. In the gel bands (Figure 9) which were selected for this analysis, one unique protein was identified in the SEA preparation corresponding to a molecular mass of approximately 27 kDa.

The samples were separated by SDS-PAGE and protein bands were excised and further analyzed by MALDI-TOF MS. The separation of proteins according to their size could be an interesting approach when aiming for a digestion method. Furthermore, stable proteins with an intrinsic resistance to proteolytic degradation (e.g. SEA) should be subjected to the digestion system with typical pre-treatment procedures with sulfhydryl reducing agents, for example DTT. The toxic activity of SEA has been also reported to be resistant to several proteolytic enzymes, for example chymotrypsin and trypsin, but can be destroyed by papain, pepsin, and pronase (Ezepchuk et al., 1982).

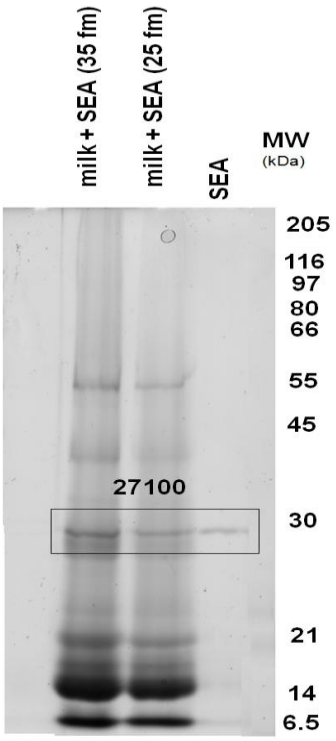

Figure 9. Coomassie blue-stained gel with different amounts of SEA.

Reductant disrupts disulfide bonds between cysteine residues, this promotes unfolding of proteins and enables analysis of single subunits of enterotoxin A. According to Callahan *et al.* (2006), reduction with DTT and alkylation with iodoacetamide before digestion was useful in the observation of several individual peptides including peptides linked by an unreduced cysteine bridge, which is present in the studied enterotoxin. Crystal structures of SEA have been studied by several authors (Schad *et al.,* 1995; Sundstrom *et al.,* 1996; Svensson *et al.,* 1997). The most typical two packed domains found in SEs are:

•& a β-barrel globular domain (residues 31–116, domain 1) which is capped by an α-helix and contains a disulfide bridge between residues 96 and 106; and

• & a C-terminal globular domain (residues 117–233, domain 2) based on a "β-grasp" motif with an α-helix packed against the β-sheet.

The two domains are linked by a long, solvent-accessible α-helix that diagonally spans the centre of the molecule. The N-terminal tail (residues 1–30) comes back to the surface of the "b-grasp" motif in domain 2.

On the other hand, the advantage of this approach is that peptide separation and detection by MALDI-TOF MS is relatively straightforward and sensitive. Peptide mass fingerprinting (PMF) is the most used technique for protein identification. The accuracy of peptide mass-to-charge (m/z) ratios obtained from coupled matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry enables identification of unknown proteins by matching of the obtained peptide masses with the theoretical peptide masses of proteins in a database, for example the Swiss-Prot database used in our study. Analysis of tryptic fragments results in definitive identification of the peptide and, by extension, the protein from which is derived. There are 23 different types of SEs and some authors have studied the amino acid sequence homology between them. Balaban *et al.* (2000) indicated that SEA was closely related with other toxins with 51–81% amino acid homology. To avoid the possibility of obtaining false positives, we

selected peptides exclusive to SEA (some fragments of the SEA's amino acid sequence do not have any homology with other toxins and we included these fragments as an important means of avoiding misidentification of SEA in the presence of other toxins). Finally, digestion of the protein shifts the analytical target from one molecular weight range to another, which offers opportunities to use cleanup strategies for peptides that cannot otherwise be used for proteins. In our results, the MALDI mass spectra were compared with those from theoretical trypsin digests of the SEA standard in order to identify the resulting peaks. Maps of the peptides which were identified for SEA are shown in Figure 10.

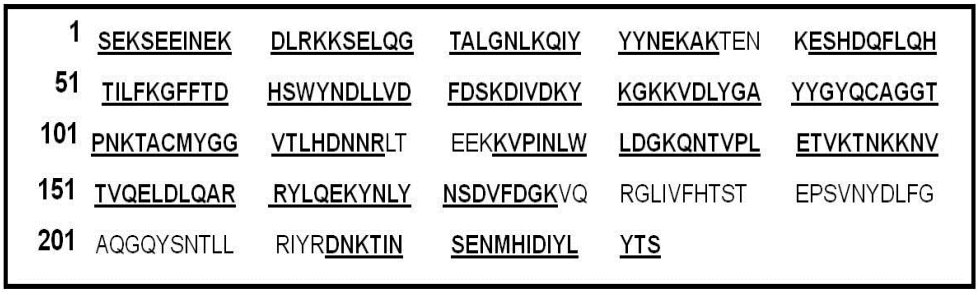

Figure 10. Identification of *Staphylococcus* enterotoxin A peptides (underlined) after tryptic digestion and peptide mass fingerprint from band excised from one-dimensional electrophoresis.

Table 30 summarizes the results of N-terminal digestion of the peptide fragments after trypsin digestion. The criteria used to accept protein identification were based on molecular weight search (MOWSE) score, the percentage of the sequence coverage, the number of matched peptides, and their length (Caruso *et al.,* 2009). Peptide mass tolerance was set to 1.2 Da, allowing two

missed cleavages per peptide. Cysteine carboxymethylation was considered as variable modification. All the searches were performed without constraining the molecular weight (Mr) and pI of the proteins, also without any taxonomic specifications. Positive protein identifications were at least five matched peptides and their length was at minimum five amino acids. These were considered according to the statistics of the software used, and the assignments were also checked manually. The results of these digestions show that under the conditions described above most of the more intense peaks appearing in the mass spectra can be assigned to peptide fragments of the digested protein (Figure 11).

Nineteen peptides (range 800–2400 Da) were identified as products of trypsin cleavage of the SEA standard with a score of 204 and 73% coverage of the protein sequence (Figure 11A), whereas thirteen peptides were revealed for SEA extracted from milk with a score of 148 and 58% sequence coverage obtained (Figure 11B). By using this strategy the studied enterotoxin spot was successfully identified. According to Godovac-Zimmermann *et al.* (2001), the ability to analyze increased numbers of peptides corresponding to higher sequence coverage is essential both to improve the reliability of protein identification and for more complete characterization of protein primary structure.

Such proteomic approaches enabled us to identify 81% of the SEA sequence; the remaining 19% escaped MALDI identification, presumably because of more thorough digestion of the primary peptides into oligopeptides and free amino acids.

Table 30. MALDI-TOF MS assignments of peptides after tryptic in-gel digestion from *Staphylococcus aureus* enterotoxin A[a].

Sequence range	$[M + H]^+_{calculated}$	$[M + H]^+_{observed}$	Peptide sequence
1-10	1192.569	1193.625	SEKSEEINEK
4-13	1233.320	1234.682	SEEINEKDLR
14-27	1486.851	1486.852	KKSELQGTALGNLK
15-27	1358.763	1358.760	KSELQGTALGNLK
16-27	1230.669	1230.694	SELQGTALGNLK
28-35	1120.531	1120.555	QIYYYNEK
28-37	1319.663	1320.716	QIYYYNEKAK
42-55	1742.886	1742.936	ESHDQFLQHTILFK
56-74	2306.040	2306.164	GFFTD HSWYNDLLVDFDSK
75-83	1065.594	1066.143	DIVDKYKGK
84-103	2225.033	2225.066	KVDLYGAYYGYQC*AGGTPNK
104-118	1651.731	1651.745	TACMYGGVTLHDNNR
104-118	1708.753	1708.798	TAC*MYGGVTLHDNNR
124-134	1282.751	1282.758	KVPINLWLDGK
135-144	1128.626	1128.661	QNTVPLETVK
135-147	1471.812	1472.660	QNTVPLETVKTNK
148-160	1513.833	1513.873	KNVTVQELDLQAR
149-160	1385.738	1385.748	NVTVQELDLQAR
161-166	836.463	836.587	RYLQEK
167-178	1434.654	1434.678	YNLYNSDVFDGK
215-233	2271.060	2272.042	DNKTINSENMHIDIYLYTS
218-233	1913.895	1913.946	TINSENMHIDIYLYTS

[a] Spot corresponding at MW of 27.1 kDa, see also Figure 9.

*Cysteines treated with iodoacetamide.

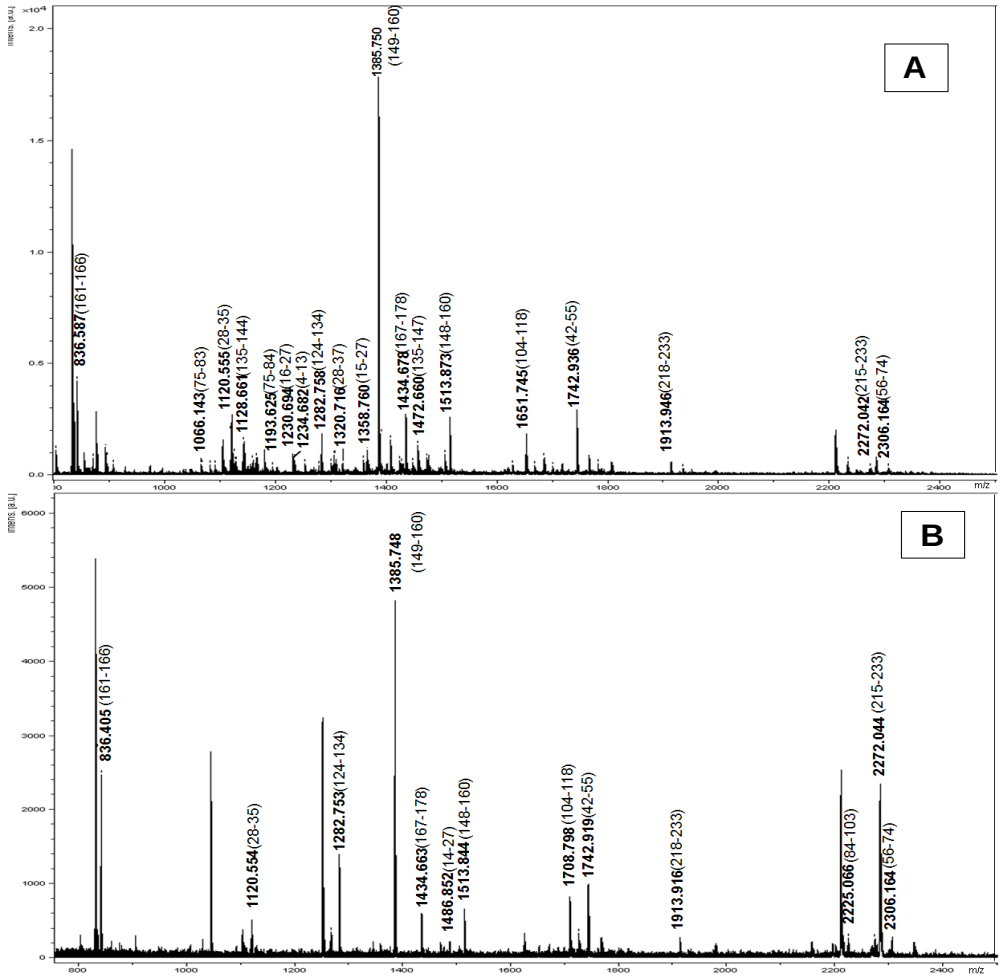

Figure 11. Peptide-matching methods demonstrated for 15 fmol SEA applied to a one-dimensional polyacrylamide gel. MALDI-TOF mass spectrometric fingerprint obtained by in-gel tryptic digestion of (A) SEA standard and (B) SEA isolated from milk, from spot corresponding to a MW of 27.1 kDa. Numbers in the mass spectrum give precise m/z values for the detected peptide ion signals, and the automatically identified corresponding amino acid positions are indicated in parentheses.

In Figure 11B, peptides 104-118 (TACMYGGVTLHDNNR) are detected, with Metl07-Thr108 being located near the cysteine

loop. According to Alakhov *et al.*, a substantial decrease in the mitogenic effect of the toxin was observed when both amino acids were digested (Alakhov *et al.,* 1992). Mamone *et al.* (2009) indicated that several foods, including dairy foods, are complex in their analysis because of a highly complex matrix of proteins, lipids, carbohydrates, and many other molecular species which interfere with detection of the toxin of *S. aureus* (present at ppb levels). In fact, Ferranti (2005) analyzed SEA and SEB of *S. aureus*, together with Shiga-like toxins produced by *Escherichia coli* O157:H7, in two Italian cheeses (Grana Padano and Pecorino Romano) with a procedure which combined a proteomic approach with immunochemical, chromatographic, and electrophoretic techniques, and tandem MS analysis. Callahan *et al.* (2006) analyzed SEB in a model food matrix (apple juice) but not milk. This group suggested that the results obtained show that although it was probably generally applicable to food matrixes with low concentrations of soluble proteins, there are still difficulties with high-protein matrixes such as milk. However, our procedure solves this problem, and this indicates that the method proposed is easy and efficient for these studied compound and matrix. The results of this study show that MALDI-TOF MS analysis combined with SDS-PAGE is a rapid and simple approach.

5.4. Quantitation of Staphylococcal Enterotoxin B by HPLC-DAD

5.4.1. Materials and methods

5.4.1.1. Food samples

A total of 20 milk samples were collected from university restaurants in the final stages of preparation. Milk was studied to detect the presence of *S. aureus* strains and their capacity to produce enterotoxins. Samples were collected randomly and aseptically in sterile bags and bottles. Milk samples, (25ml) were suspended in 225 ml of BPW.

5.4.1.2. Bacterial identification

Serial decimal dilutions were prepared from the initial dilution. Each of the different dilutions (0.1 ml) was transferred onto the surface of plates containing BP agar supplemented with tellurite and egg yolk emulsion. Plates were incubated at 37 ºC for 24-48h. Suspected colonies were subjected to Gram staining, examined microscopically, and identified with the API Staph system. To increase the production of enterotoxins, isolated staphylococcal strains were prepared by inoculating seven colonies from BP agar into 10 ml of tryptone soya broth. After 18 to 24h of growth at 37 ºC with shaking, the cultures were centrifuged and the supernatant was filtered with 0.22-µm hydrophilic regenerated cellulose filter membranes, and the filtrate was retained for an assay of the toxin content.

5.4.1.3. HPLC-DAD analysis

Bacteria cultures identified as *S. aureus* were analyzed with HPLC-DAD. Culture filtrates were concentrated and desalted using 10k NMWL Millipore centrifugal filter units. Samples were centrifuged at 9000 rpm and washed twice with 0.1% TFA in ACN. HPLC-DAD analysis was performed using a Shimadzu LC system equipped with LC-10AD pumps and a diode array detector (DAD) from Shimadzu (Japan). A Jupiter C18 reversed-phase analytical column (150 x 2mm, 3µm) Phenomenex column was used with a flow rate of 0.2 ml/min. The volume injected of standards and sample solutions was 20 µl. Solvent A was 0.1% TFA in water, and solvent B was 0.1% TFA in ACN. HPLC conditions were set up using a gradient of 30 min that started at 20% B with a linear gradient of 20-40% B in 10 min, then changed to 60% B at 20 minutes, and the gradient backs to 20% B at 30 minutes. SEB was detected at 205nm and 23.5 min. Enterotoxin identification was performed by comparing retention time and UV spectra of purified extracted samples to pure standards.

5.4.2. Results and discussion

In order to optimize the developed HPLC-DAD method, limit of detection (LOD) and limit of quantification (LOQ) were calculated according to s/n=3 and s/n=10, respectively. LOD and LOQ values were 0.5µg/mL and 1µg/mL, respectively. The technique of standard additions was used to calculate the recovery of this method. Non enterotoxigenic bacteria cultures were added

with SEB standard solution at five levels in a range of 1-50 µg/ml in triplicate. Mean recoveries of fortified cultures ranged from 89.1±2.7% to 101.6±2.1% for concentrations tested. Standard and fortified matrix curves showed a good linearity; coefficients of correlation were (r^2) greater than 0.997 and no matrix effect was detected. For repeatability and reproducibility, five series of samples spiked at 5µg/ml were compared with SEB standards signal. The RSD obtained for intraday variation (n=5) was 3.1%. The inter-day variation showed a RSD value of 7.3%. These values were lower than 20% confirming the good reproducibility and repeatability of this technique (European Union, 2002).

Out of 20 milk samples examined, 9 (45%) revealed typical colonies of *S. aureus* on BP agar. The high percentage of contaminated milk samples found in this work is according with previous studies in milk samples (Sospedra *et al.,* 2009). All analyzed samples were collected from restaurants and possible infringements of hygienic practices in the handling and cleaning of the milk containers can also contribute to increase the microbial contamination of these kind of samples. Due to its high level of nutrients, milk samples provide a suitable growth medium for several bacteria. Only 35% of the isolates were identified as *S. aureus* species by the coagulase test API Staph system. One of these isolates demonstrated to be enterotoxigenic.

S. aureus can produce enterotoxins in milk samples usually because the food has not been kept hot enough or cold enough (Scott *et al.,* 2007; Mattick *et al.,* 2003). Staphylococci are present

in the nasal passages and throats and on the air and skin of approximately 50% of healthy individuals. Although food handlers are frequently implicated in the transmission of this pathogen to food, equipment and environmental surfaces can also be sources of contamination with *S. aureus* (Bhatia *et al.,* 2007).

Huong *et al.* (2010) also studied the presence of *S. aureus* and their toxins in ready-to eat foods and according to our results (45% of the samples with typical colonies of *S. aureus* on BP agar and 35% confirmed by API test), they found that around 35% of the studied milk samples were contaminated by *S. aureus*.

In the recent years several works about the presence of staphylococcal enterotoxins in milk have been developed. Generally, the percentage of enterotoxigenic strains among *S. aureus* isolates is variable as it differs from one food to another and from one report to another. The percentage of enterotoxigenic *S. aureus* strains (5%) found in our study is similar to those reported by other workers for the same and different food items; in Turkey for bovine milk (5.6%) (Boynukara *et al.,* 2008); In 4.8% of isolates from fresh and processed meat (Al-Tarazi *et al.,* 2009) or in the work of Soriano *et al.* (2002b) who found enterotoxigenic *S. aureus* strains in 4% of 504 food samples analyzed. But it differs from the following studies; the 66% reported by Marthenge and Ombui (2007) where 200 raw milk, 100 beef carcass swabs, 50 minced meat samples and 50 chicken carcasses were examined. Also Hwang *et al.* (2007) founded

enterotoxigenic *S. aureus* strains in 43% of their pork and chicken meat samples.

Variable percentages are recorded for different food items in different countries. The variability of food examined could explain the diversity of enterotoxigenic prevalence among *S. aureus* and thus demonstrates the specificity of food (intrinsic factors) and its environment (extrinsic factors) in creating more favorable conditions for *S. aureus* to multiply and producing its enterotoxins.

SEB was the enterotoxin produced by enterotoxigenic strain isolated and it was confirmed by HPLC-DAD (Figure 12).

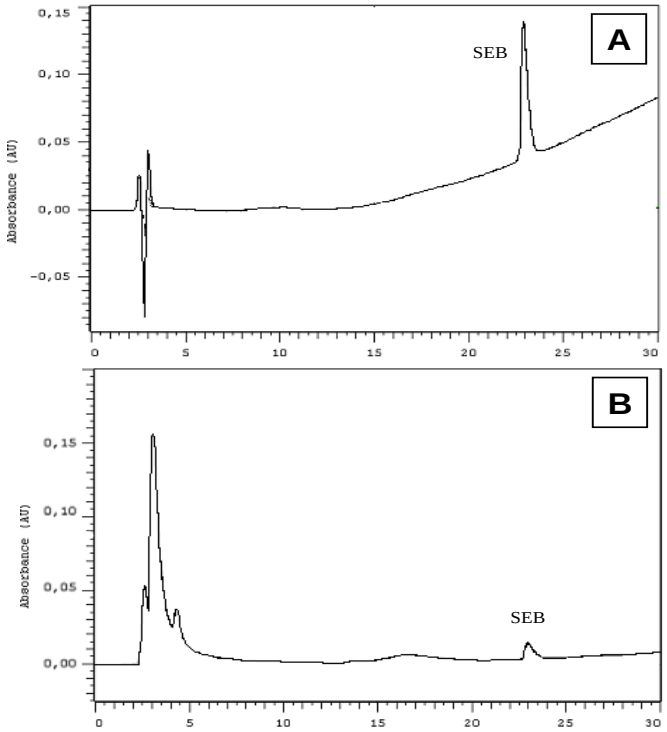

Figure 12. HPLC-DAD chromatograms; (A) bacteria culture spiked by intact SEB standard (10µg/mL) (B) enterotoxin B-positive culture isolated from milk.

Like us, the studies from other authors have revealed the presence of SEB in milk samples. Normanno *et al.* (2007) detected several SEs (A, B, C and D) in 6 samples of milk and dairy products from Italy by SET-RPLA kit. Huong *et al.* (2010) found SEA, SEB and SEC in 52.9% of the ready-to eat milk samples analyzed also using SET-RPLA. However, none of these studies reflect the quantity of toxin found. Our results reflect that the quantity of SEB found produced by *S. aureus* strain was 3.6 µg/mL (Table 31).

Table 31. Prevalence of *S. aureus* in milk samples and incidence of enterotoxigenic strains.

Food	Nº of samples	Nº of samples shows typical colonies on BPA	Nº of samples positive for *S. aureus* by coagulase test	Nº of enterotoxigenic positives	SEs detected by HPLC-DAD (µg/ml)
Milk collected from restaurants	**20**	**9**	**7**	**1**	**B (3.6)**

SET-RPLA method has several advantages at present, including high specificity, simplicity and economy. One of the disadvantages of this test is that is a semiquantitative method; some works tried to calculate the recovery of toxin from foods by the concentration of toxin detected with the RPLA method and the volume of the supernatant fluid (Park and Szabo, 1986). However, the concentration of toxin by this method is calculated discontinuously by the toxin titer of a sample and the sensitivity of toxin. For this reason, it is thought impossible to evaluate the

exact recovery of toxin from a sample by SET-RPLA. On the other hand, one of the most important advantages of HPLC-DAD is that this method allows the quantification of the enterotoxin detected.

Other immunological assays have been applied to SEB´s detection, such immunodiffusion assays, radioimmunoassay and enzymelinked immunosorbent assays. However, these techniques have some limitations like the difficult on the handling and disposal of radioactive compounds and the low binding efficiency of the radioactive labeled toxin (Ler *et al.,* 2006). The relevance of immunological approaches is still under discussion because can also origin false positives (Berdal *et al.,* 1981). The proposed technique allows a rapid identification and quantification of the whole protein based on its retention time and UV spectra compared with a standard to avoid the possibility of obtaining false positives. Frequently, immunological methods require also time consuming incubation steps, long time incubation is necessary to obtain reliable results and this fact represents a strong drawback for wide diagnostic applications (Morissette *et al.,* 1991). It occurs also with SET-RPLA test, according to the manufacturer, the plate must be left on a vibration-free surface at room temperature for 20 to 24 hours and then examine each well in each row for agglutination.

Other advantage of HPLC-DAD is that using this method, the run time takes only 30 min which is much less than the time needed by other techniques used for SEB´s detection.

During recent years, the in-gel enzymatic digestion procedure followed by mass spectrometric analysis has been used by several authors to increase the yield and recovery of the peptides. Nedelkov *et al.* (2000) detected SEB in milk and mushrooms by use of a combination of antibody extraction and MALDI–MS. Kawano *et al.* (2000) showed that a variety of proteins produced by *S. aureus*, including enterotoxins, could be characterized by LC-MS. Our group also developed a procedure for detection of SEA in milk by use of matrix assisted laser desorption/ionization-time of flight (MALDI–TOF) (Sospedra *et al.,* 2011).

The use of mass spectrometry techniques provides good sensitivity and specificity but its main drawback remains high cost and sophisticated instrumentation. Most of the techniques used recently for SEs´ detection are also time-consuming because they include a tryptic cleavage step for protein digestion or an incubation step.

However, the results obtained suggest that HPLC-DAD could be applied to bacteria culture isolates from food samples for the detection of SEB. The proposed technique allows a rapid identification and quantification of the whole protein based on its retention time and UV spectra compared with a standard to avoid the possibility of obtaining false positives.

5.5. Simoultaneous Quantitation of Staphylococcal Enterotoxins types A and B by Liquid Chromatography/Mass Spectrometry

5.5.1. Materials and Methods

5.5.1.1. Chemicals and reagents

HPLC grade acetonitrile and analytical-grade formic acid were purchased from Merk (Darmstadt, Germany). Deionised water for LC mobile phase was prepared by reverse osmosis with a Milli-Q water purification system (Millipore, Molsheim, France). Phenex-RC filters were supplied by Phenomenex (Madrid, Spain). SEA and SEB were purchased from Sigma (Sigma Chemicals, St. Louis, USA).

5.5.1.2. Sample preparation

Primary stock solutions of SEA and SEB (1 mg/mL) were prepared in water and stored at -18 ºC until use, according to Callahan *et al.* (2006). Stock solutions were further diluted with water to obtain working solutions at seven different concentrations (0.01, 0.05, 0.1, 0.5, 1, 5 and 10 µg/mL).

To prepare fortified samples, 900 µL of milk, orange or apple juice "blank" samples (sample in which was corroborated, before the analysis, that no toxin was present) were spiked with working solutions (100 µL) at five different levels to provide solutions with the following concentrations: 0.05, 0.1, 0.5, 1 and 5µg/mL. Spiked samples were left to incubate for 30 min at room temperature to allow interaction between toxins and food matrix. Every different concentration was prepared in triplicate in five different days and

was analysed in five independent runs. Before the analysis, apple juice samples were filtered with hydrophilic regenerated cellulose filter membranes. Orange juice samples have been filtrated like apple juice with the exception that particulate matter was centrifuged from the sample prior to filtration. Due to high fat and protein content of the milk, which can interfere with the analysis, the extraction procedure of SEs from milk was done by the following method, based on a previous study (Sospedra *et al.,* 2011). A sample of skimmed cows' milk was spiked with different concentrations of enterotoxin. This milk (1 mL) was purified. Milk protein precipitation was performed with 200 μL dichloromethane and 100 μL water containing 5% acetic acid at pH 4.5 before centrifugation for 15 min at 5,000 rpm. The supernatant was collected and the desired proteins are thus free from interferences from casein and fats.

For the analysis of real samples, eight samples of fresh orange juices and ten milk samples were collected from university food services.

5.5.1.3. LC-ES/MS/SIR instrumentation and conditions

Separation by LC was performed using a C4 reversed-phase analytical column (Jupiter 300Å, 250 x 2.00 mm, 5μm Phenomenex), preceded by a security guard cartridge C4 (2 mm × 3 mm I.D.), with a flow rate of 0.2 ml/min. The volume injected of standards and sample solutions was 20 μl. Solvent A was 0.5% acetic acid in water, and solvent B was 0.5% acetic acid in ACN.

Separation of enterotoxin proteins was carried out in a gradient of 13 min that started at 0% B with a linear gradient of 0-30% B in 9 min, then changed to 20% B in one minute, and in 3 min the gradient backs to 0% B. Then, flow remains during 5 min at the initial conditions for reequilibration. The triple quadrupole mass spectrometry detector (QqQ) was equipped with an LC Alliance 2695 system (Waters, Milford, MA, USA) that included an autosampler and a quaternary pump. A QqQ mass spectrometer Quattro LC from Micromass (Manchester, UK) equipped with pneumatically assisted electrospray probe, a Z-spray interface and Mass Lynx software was used for MS analyses. Analysis was performed in positive ion mode. The ESI source values were capillary voltage, 3.00 kV; extractor, 3 V; RF lens 0,2 V; source temperature, 150°C; desolvation temperature, 300°C; desolvation gas (nitrogen 99.99% purity) flow, 600 l/h. Dwell time were set to 0.2 s. The mass spectrometer was operated in scan and selective ion recording (SIR) modes. Mass spectra were scanned from 600 to 1300 Da at a scan cycle of 1s per scan. Cone voltages and collision energies were optimized for each analyte by continuous infusion of a standard solution (5μg/ml) via syringe pump at a flow rate 20 μl/min. The most abundant charged ions for SEA and SEB were chosen for the selective ion recording. Chromatograms and mass spectra were analyzed using the Waters/Micromass Mass Lynx NT Ver. 4.1 data system.

5.5.2. Results and discussion

We applied LC-ESI/MS for rapid identification of SEA and SEB, which have been analyzed with satisfactory results as intact proteins. The mass spectra obtained of both whole proteins are shown in Figure 13. The spectrum of each enterotoxin is characterized by the extensive distribution of charge states typical of electrospray MS of proteins. Under the MS conditions used, the maximum of the envelope of the protonated species was at 29 charges for SEA and 31 for SEB.

In order to validate the developed procedure, repeatability, reproducibility, as well as limits of detection (LODs) and limits of quantification (LOQs) were experimentally calculated. The LODs and LOQs were based on minimum amount of target analyte that produced a chromatogram peak with a signal-to-noise ratio of 3 and 10 times the background chromatographic noise, respectively. Estimated values of LODs were in the range of 0.5 and 0.2ng for SEA and SEB respectively, whereas LOQ values were in the range of 1ng for both enterotoxins. These values were determined by SIR mode; the error allowed in the average mass at this level was 0.5 Da.

Intra and inter-day precisions of the developed analytical method were studied by assaying five consecutive times within the day (intra-day precision), and for five consecutive days (inter-day precision) for each analyzed compound. The values of repeatability and reproducibility were determined by calculating the corresponding relative standard deviations (RSD) in the same and different days respectively (Table 32).

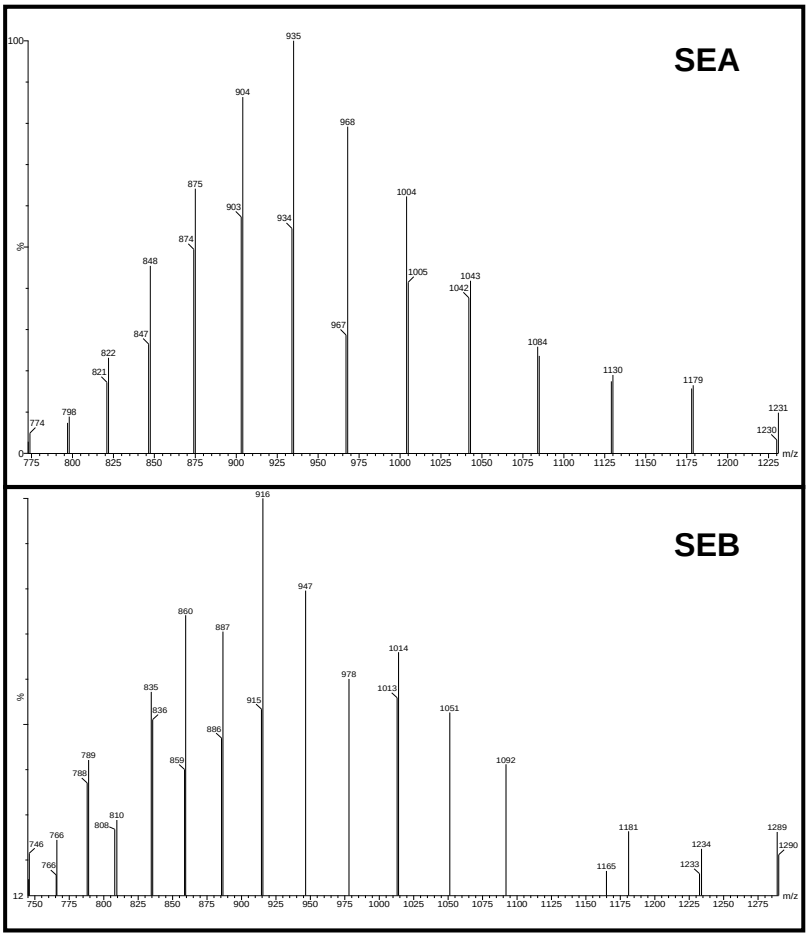

Figure 13. Mass spectra of SEA and SEB with ion abundance profile.

The linearity of the method was studied by analyzing the standard solutions and the different matrices. Standard curves for the three food matrices studied were constructed on five different working days, at five concentrations ranging from 1-100 ng, for SEA and SEB. Satisfactory linearity was obtained when the correlation coefficient (R^2) was higher than 0.997 based on measurement of the analyte peak areas. The absolute recoveries

were determined by comparing the mass spectrometry response of spiked samples with calibration standards.

Table 32. Repeatability and reproducibility for SEA and SEB.

	Concentrations studied	Repeatability (RSD, %) (n=5)	Reproducibility (RSD,%) (5 different days)
SEA	0,05	10,53	5,83
	0,1	2,32	2,63
	0,5	3,01	5,99
	1	1,78	5,20
	5	4,17	5,36
SEB	0,05	3,65	7,01
	0,1	9,34	4,99
	0,5	5,54	3,93
	1	1,34	1,93
	5	3,02	4,25

To evaluate matrix effects, the signal suppression enhancement (SSE) for each analyte in each matrix was studied (Figure 14). The SSE was defined as the percentage of the matrix matched calibration slope divided by the slope of the standard calibration in solvent. To verify the absence of interfering species around the retention time of the analytes, blank samples were analyzed. To differentiate between extraction efficiency and matrix-induced signal suppression/enhancement, the slope ratios of the linear calibration functions were calculated, and the signal suppression/enhancement (SSE) due to matrix effects was determined (Table 33).

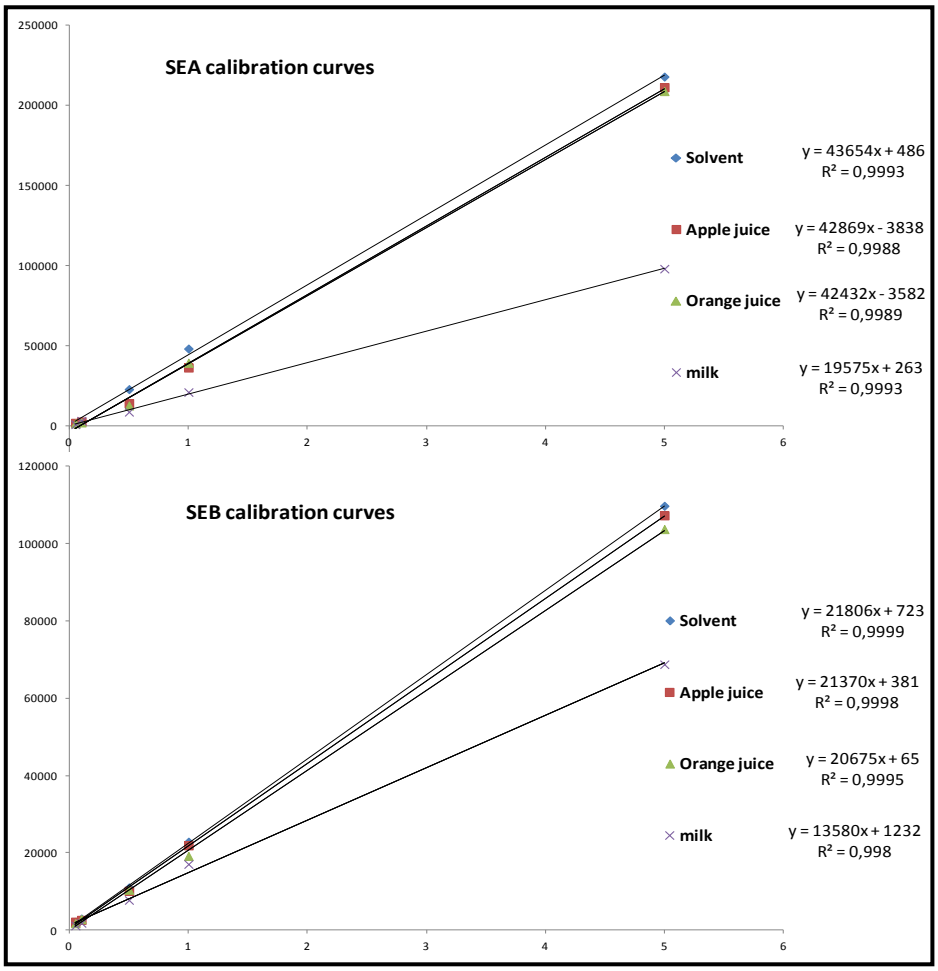

Figure 14. Graphic representation of calibration curves of SEA and SEB in solvent and in matrices (apple and orange juices and milk).

Under optimized LC and MS conditions, no interferences from endogenous compounds were found in orange or apple juices blank samples at the expected retention time as evidenced by the slope ratios, which were within 10% of the slope ratio=100% (95–98%), and the high linearity (R^2 > 0.9988) of the calibration curves. Despite the extraction processes had eliminated several interfering

components as caseins, in complex matrix as is milk can remain some compounds that may lead to inaccurate results.

Table 33. Linear regression parameters of calibration curves of SEA and SEB and calculation of signal suppression/enhancement (SSE) in solvent and in matrices (apple and orange juices and milk).

Enterotoxin	Matrix	Calibration range (µg/mL)	Standard calibration curve			*SEE (%)
			Slope	*y*-intercept	R^2	
SEA	Solvent	0,05-5	43654	486	0,9993	-
	Apple juice	0,05-5	42869	3838	0,9988	98
	Orange juice	0,05-5	42432	3582	0,9989	97
	Milk	0,05-5	19575	263	0,9993	45
SEB	Solvent	0,05-5	21806	723	0,9999	-
	Apple juice	0,05-5	21370	881	0,9998	98
	Orange juice	0,05-5	20675	65	0,9998	95
	Milk	0,05-5	13580	1232	0,9997	62

* SSE= (slope matrix-matched calibration/slope standard calibration in solvent) ×100%

Consequently, quantification was performed with the external calibration using standards in pure solvent for fruit juices and using matrix matched calibration for milk. SEs´ retention times were 6.29 ± 0.02 and 6.66 ± 0.03 for SEA and SEB, respectively (Figure 15).

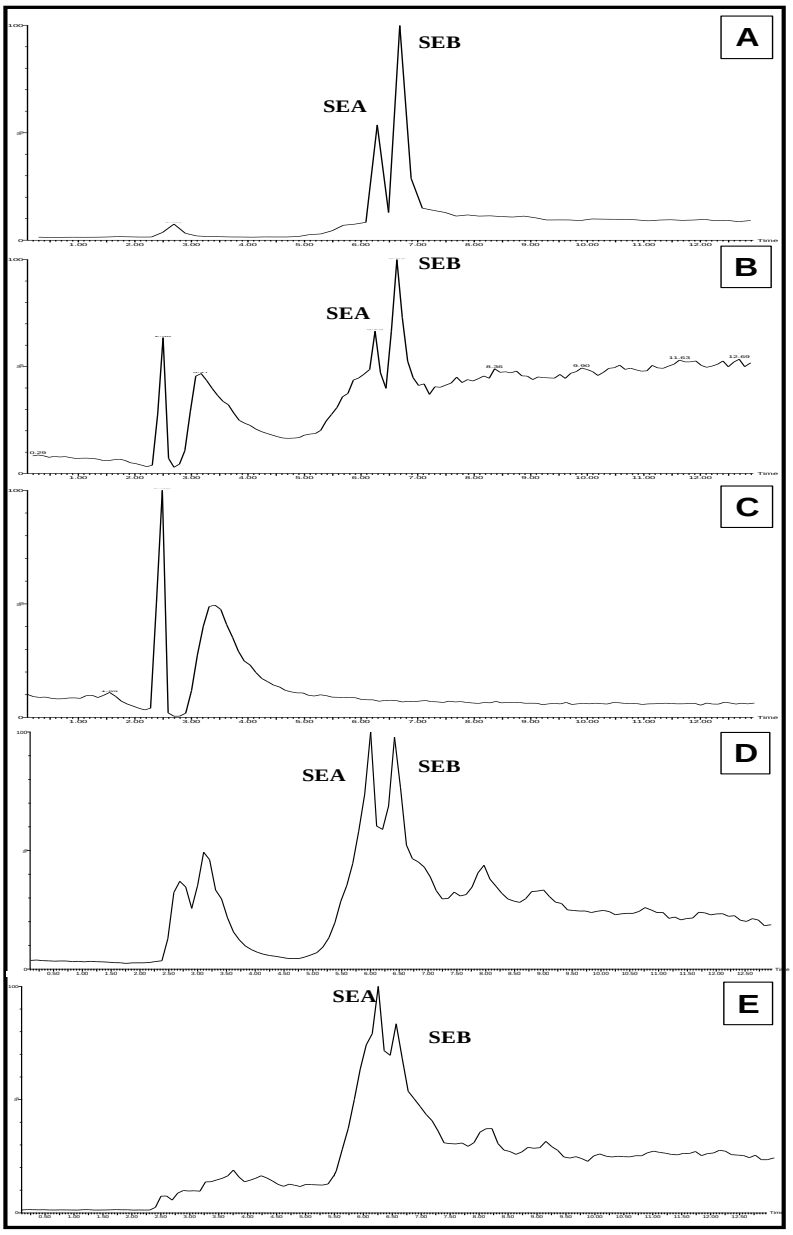

Figure 15. LC–MS chromatograms; (A) SEA and SEB standard, (B) extract of apple juice fortified with of SEA and SEB enterotoxins respectively, (C) extract of juice sample non-fortified, (D) and (E) extracts of orange juice and milk, respectively, fortified with SEA and SEB.

The recoveries values obtained for apple juice samples ranged from 87 to 99% (Table 34). Recoveries obtained for milk and fresh orange juice samples were lower than recoveries for apple juice due to high content of fat, proteins and other compounds in these kind of matrices. The results obtained ranged from 69 to 81% and from 72 to 93% for milk and orange juice samples respectively (Table 34).

Table 34. Recoveries from SEA and SEB in spiked, apple and orange juices and milk.

	APPLE JUICE		ORANGE JUICE		MILK	
SEA added (µg/ml)	**SEA detected (µg/ml)**	**Recovery (%)**	**SEA detected (µg/ml)**	**Recovery (%)**	**SEA detected (µg/ml)**	**Recovery (%)**
0.05	0,046±0,006	92	0.038±0,006	76	0.040±0,003	81
0.1	0,094±0,001	94	0.079±0,007	79	0.080±0,004	80
0.5	0,436±0,012	87	0.445±0,008	89	0.387±0,273	77
1	0,893±0,018	89	0.876±0,020	88	0.793±0,293	79
5	4,705±0,32	94	4.631±0,076	93	3.92±0,258	78
SEB added (µg/ml)	**SEB detected (µg/ml)**	**Recovery (%)**	**SEB detected (µg/ml)**	**Recovery (%)**	**SEB detected (µg/ml)**	**Recovery (%)**
0.05	0,045±0,005	90	0.036±0,003	72	0.034±0,002	79
0.1	0,088±0,018	88	0.073±0,007	73	0.710±0,002	71
0.5	0,467±0,017	93	0.400±0,023	80	0.391±0,022	78
1	0,915±0,031	91	0.841±0,026	84	0.732±0,022	73
5	4,965±0,22	99	4.530±0,195	91	4.010±0,225	81

S. aureus is commonly detected in foods and, among the products involved in food poisoning caused by SEs, milk is one of the most frequently contaminated.

The percentage of enterotoxigenic *S. aureus* strains (5%) found in our previous study evidenced that milk is frequently contaminated by SEs and these results are similar to those reported by other workers for the same and different food items; Boynukara *et al,* (2008) detected SEs in 5,6% of bovine milk samples. Fruit juices have also been studied for the presence of staphylococcal enterotoxins. Kaur *et al.* (2006) analyzed freshly squeezed kinnow-mandarin and carrot juices. They found SEB and SEC in 9% of samples collected. Moon *et al.* (2007) studied the presence of *S. aureus* organisms that produced SEs in food samples and they detected that 8% of vegetables samples were contaminated by enterotoxigenic *S. aureus* and the enterotoxin most frequently founded was SEA. Despite these data, none of the samples of orange juice and milk tested demonstrated to be contaminated by enterotoxins A and B.

Currently methods for toxins analysis are based on either a biological approach using immunological techniques or a chemical approach using spectrometric techniques. With immunological methods long time incubation is necessary to obtain reliable results and this fact represents a strong drawback for wide diagnostic applications (Stephan *et al.,* 2001). Determination and identification of enterotoxins using traditional biochemical tests are time consuming and other proteins excreted by *S. aureus* can cause false positives results (Berdal *et al.,* 1981). The proposed method allows specific detection of SEA and SEB with total discrimination from other types of SEs. To avoid the

possibility of obtaining false positives, SEA and SEB identification is based on the spectra obtained, which depend on the multiple charges of the protonated proteins. With the LC-ESI/MS technique used, molecular mass determination will be uncompromised by fragment ions. For molecules that show ability for multiple charging, as are enterotoxins, precision is enhanced by the possibility for multiple mass measurements from a single mass spectrum. An important feature of ES is that essentially no fragmentation accompanies ionization of peptides or proteins, like demonstrated Fenn *et al.* (1989). This group was the first to describe the analysis of samples containing polymers, peptides and proteins with molecular mass beyond 20000 using an electrospray (ES) interface.

In the work of Bennet *et al.* (1992) with immunological assays, SEA and SED were found to be undetectable (loss of serological recognition) but still active after heat treatment. Consequently, false negatives may also result. Although immunological assays are quicker and cheaper, the relevance of the immunological approach is still under discussion.

In the last years, the in-gel enzymatic digestion procedure followed by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry analysis was used to increase the yield and recovery of the peptides by several authors (Sospedra *et al.,* 2011; Bernardo *et al.,* 2002; Pocsfalvi *et al.,* 2008). Using these methods, it is possible to achieve a high sensitivity and specificity. However, the process is tedious and time

consuming. Enzymatic digestion takes a long time and, with the difficult of an accurate quantification of the total toxin after digestion, are the main disadvantages of these techniques. Furthermore, the presented method detects the whole proteins and it improves the time of analysis. This fact allows a more accurate quantification which means a great advantage in foodborne diseases analysis. One of the most important advantages of LC-ESI/MS is that this method allows a quantification of the enterotoxins detected.

The major problem with identifying enterotoxins in foods is that minute concentrations are sufficient to cause food poisoning. The threshold amount of enterotoxin for causing illness in humans is not known. However, information from food poisoning outbreaks and human challenge studies indicates that individuals experiencing illness probably consumed at least 100 ng of enterotoxin A (Hennekinne *et al.* 2009). SEB has an infective/lethal dose of 0.02 μg/kg (Walt and Franz, 2000). Hennekinne *et al.* (2009) detected SEA at levels of 1.3± 0.2 ng/g by a quantitative MS assay using specific isotope-labelled [^{13}C] and [^{15}N] SEA standards in coconut pearls. Portocarrero *et al.* (2002) arrived at levels of 1ng/ml of SEA, SEB, SEC and SED stock solutions with TECRA kit. Coupling immunomagnetic separation on magnetic beads with matrix-assisted laser desorption ionization-time of flight mass spectrometry Schlosser *et al.* (2007) obtained a LOD of 2 ng for SEB. Dupuis *et al.* (2008) developed a strategy to identify and quantify SEA in food matrices with a limit of 2.5±0.2ng/g. Their

method was based in a combination of immunocapture and protein standard absolute quantification. Callahan *et al.* (2006) also worked with SEB in apple juice and their mass spectrometry studies were performed on a Micromass QTOF Micro quadrupole time-of-flight (QTOF). They concluded that whole protein analysis methods in complex mixtures such as bacterial lysates, does not allow for detection of SEB. However, the purposed method can detect successfully both enterotoxins, SEA and SEB in several food matrices at levels around 0.05µg/mL.

LC-ESI/MS method is a good tool for analysis of both exotoxins that are the most prevalent compounds in staphylococcal food poisoning worldwide; this technique is less time-consuming because avoid the necessity of preliminary steps to isolate the bacteria from food. Under the proposed conditions, SEA and SEB can be directly detected from milk and other food samples. On comparing our results with other studies, it can be seen that the use of a sensitive and moderately time consuming method to detect SEA and SEB may be an advantage in microbial quality assurance of food products.

5.6. Staphylococcal enterotoxins production by enterotoxigenic *S. aureus* strains isolated from food samples

5.6.1. Materials and methods

5.6.1.1. Reagents

BPW CM 509, BP agar CM 275, Tryptone Soya Broth CM 129, Tellurite reagent, Egg Yolk Emulsion SR 275 and SET-RPLA kit were supplied by Oxoid, Unipath, Hampshire, UK. API Staph system was from BioMérieux, Marcy l´Etoile, France.

HPLC grade acetonitrile was purchased from Merk (Darmstadt, Germany) and analytical-grade trifluoroacetic acid (TFA) was supplied by Sigma-Aldrich (St. Luis, USA). Deionised water for LC mobile phase was prepared by reverse osmosis with a Milli-Q water purification system (Millipore, Molsheim, France). Solvents and water were degassed for 20 min using a Branson 5200 (Branson Ultrasonic Corporation, Connecticut, USA) ultrasonic bath. 0.22-µm hydrophilic regenerated cellulose filter membranes Phenex-RC and Millipore 10K centrifugal filters were supplied by Phenomenex (Madrid, Spain) and Millipore (Molsheim, France), respectively. The stock standard solutions of SEA and SEB were purchased from Sigma-Aldrich (St. Luis, USA).

5.6.1.2. Food samples

Among the foods implicated in SFP, milk, dairy products, egg products and meats, especially handled foods, play an important role since enterotoxigenic strains of *S. aureus* have been

frequently isolated in them (De Buyser *et al.*, 2001; Normanno *et al.,* 2005).

A total of 120 samples including meat (pork, chicken and beef) (n=34) milk (n=20) and egg products (spanish omelette, fried eggs and hardboiled eggs) (n=66) were collected from restaurants in the final stages of preparation. These samples were studied to detect the presence of *S. aureus* strains and their capacity to produce enterotoxins. Analyzed products include raw and processed foods that are commonly consumed in these establishments (Rico *et al.*, 2005).

5.6.1.3. Isolation and identification of *S. aureus*

Samples, (25g) were weighed into sterile stomacher bags, diluted with 225 ml of BPW, and homogenized in a stomacher (Classic, IUL, Barcelona, Spain). The samples were further diluted with buffered peptone water, and 0.1 ml portions of various dilution levels were spread on the surfaces of BP agar supplemented with tellurite and egg yolk emulsion. Plates were incubated at 37 ºC for 24-48h.

Colonies were subjected to Gram staining, examined microscopically, and identified with the API Staph system. Isolated staphylococcal strains were prepared by inoculating seven colonies from Baird Parker agar into 10 ml of tryptone soya broth. After 18 to 24h of growth at 37 ºC with shaking, the cultures were filtered with 0.22-µm hydrophilic regenerated cellulose filter

membranes, and the filtrate was retained for an assay of the toxin content.

5.6.1.4. Detection of SEs

S. aureus strains were studied for their ability to produce enterotoxins according to the SET-RPLA methodology and with the proposed method by LC-ESI/MS.

5.6.1.4.1. Reverse passive latex agglutination (RPLA)

Enterotoxin production in the filtrate was assessed with the SET-RPLA kit as directed by the manufacturer. The procedure involves four suspensions of staphylococcal enterotoxin antibody-coated latex, one each for enterotoxins A (SEA), B (SEB), C (SEC) and D (SED). Also a suspension of rabbit serum immunoglobulin G-coated latex was included.

The microtiter plates of the SET-RPLA kit were sealed with a plate sealer and shaken to mix the contents of the wells. Immediately after that, the plates were incubated at room temperature on a vibration-free surface, and the agglutination reactions were read after 20 to 24 h by holding the plates against a dark background with indirect lighting.

5.6.1.4.1. LC-ESI/MS

Enterotoxin production in the filtrate was also assessed by LC-ESI/MS to confirm the production of enterotoxins A and B and to quantify them.

Culture media were centrifuged at 5000 rpm for 5 min. After that, 1 ml of supernatant was concentrated and desalted using 10k NMWL Millipore centrifugal filter units. Samples were centrifuged at 9000 rpm and washed twice with 0.1% TFA in ACN. The extracts of the media culture were injected into the chromatographic equipment.

In order to optimize the developed LC-ESI/MS method with the extraction procedure proposed, the determination of precision, accuracy and the LOD and LOQ, were done using the matrix assisted calibration.

5.6.2. Results and discussion

5.6.2.1. Samples contaminated by *S. aureus*

Out of 120 food samples examined, 37 (30.8%) revealed typical colonies of *S. aureus* on BP agar. Only 11 (9.2%) of the isolates were identified as *S. aureus* species by the API Staph system. Four of them were meat samples whereas seven were milk samples (Table 35). None of the egg based dishes was contaminated by *S. aureus* strains.

5.6.2.2. Detection of SEs in culture supernatans

About 10% of examined food samples were contaminated by *S. aureus*, but only 3% (2 milk samples and 2 meat samples) demonstrated to be enterotoxigenic strains.

All the enterotoxigenic strains detected by SET-RPLA were also detected and confirmed by LC-ESI/MS method. However, both

methods did not identify the same enterotoxins in all samples. All the enterotoxigenic strains produced SEA but SEB was only found in 25% of the cultures tested by SET-RPLA (Figure 16) whereas LC-ESI/MS method identified SEB in 50% of the enterotoxigenic strains (Table 35).

Table 35. Distribution and prevalence of *S. aureus* in food samples and enterotoxin production.

Food		Nº of samples	Nº of samples positive for *S. aureus*	Nº positive samples for enterotoxigenic strains of *S. aureus*	SEs detected by SET-RPLA	SEs detected by LC-ESI/MS-MS (µg/ml)
Milk		20	7	2	A A	A (1.03) A (2.61), B (0.27)
Meat	Pork	12	1	1	A	A (7.20)
	Chicken	13	2	0	---	---
	Beef	9	1	1	A, B	A (0.39), B (0.15)

Since today, the presence of enterotoxigenic staphylococci and their toxins have been detected in different foods and beverages. According with literature the most prominent enterotoxin type produced by the enterotoxigenic staphylococci is SEA. The highest percentage of enterotoxigenic strains that produce SEA reported in this study (100%) is also noticed for other meat and milk samples in Germany, Italy and Spain Atanassova, et al., 2001; Normanno *et al.*, 2005; Normanno *et al.*, 2007a). Normanno *et al.* (2007b) detected several SEs (A, B, C and D) in 6 samples of milk and dairy products from Italy using SET-RPLA kit.

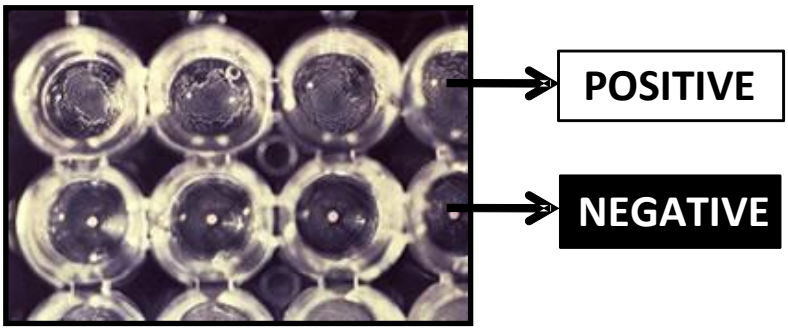

Figure 16. Image of SET-RPLA test showing agglutination and a diffuse layer on the base of the wellin for positive results and a tight button for negative results.

5.6.2.3. Quantification of SEs produced by *S. aureus* isolates

Calibration curves were performed by spiking standard solutions of both enterotoxins at five different concentrations ranging 0.05-5µg/mL to cleaned extracts of a bacteria culture of a non enterotoxigenic staphylococcal strain. LOD and LOQ were calculated according to s/n=3 and s/n=10, respectively. LOD values obtained were 0.1 and 0.05µg/mL and LOQ values were 0.1 and 0.2µg/mL for SEA and SEB respectively.

Table 36. LODs, LOQs, recovery values (%) intra- and inter-day precision of bacteria culture spiked with SEA and SEB.

	Limit of detection (LOD, µg/mL)	Limit of quantification (LOQ, µg/mL)	Concentrations studied (µg/mL)	Recovery (%)	Repeatability (RSD, %) (n=3)	Reproducibility (RSD,%) (5 different days)
SEA	0,1	0,2	0,2	84	2,32	2,63
			2	91	1,78	5,20
SEB	0,05	0,1	0,1	87	3,65	7,01
			1	101	3,02	4,25

Recovery experiments were conducted at two levels, between 0.1 and 0.2μg/mL (quantification limits, LOQs) and between 1 and 2μg/mL (10×LOQs) in triplicate and in five different days. Mean recoveries, repeatability and reproducibility values obtained are shown in Table 36 and are in agreement with current legislation (European Union, 2002).

Peaks with spectra and retention times corresponding to SEA and SEB standards were identified and the peak areas were compared with matrix assisted calibration to quantify them. Enterotoxins A and B produced in bacterial cultures were quantified by LC-ESI/MS (Figure 17) and the results obtained are shown in Table 35.

The RPLA method is semiquantitative and according to the manufacturers, the sensitivity of SET-RPLA test detecting enterotoxins is 0.5ng/ml. The concentration of the toxin by this method is calculated discontinuously by the toxin titer of a sample and the sensitivity toxin. For this reason, it is thought impossible to evaluate the exact recovery of toxin present in a sample by SET-RPLA.

The results obtained suggest that either SET-RPLA or LC-ESI/MS could be applied to culture filtrates for the detection of SEs with good correspondence of results. Although SET-RPLA represents a simple method for routine monitoring purposes, a positive result by a rapid method (RPLA) is only regarded as presumptive and must be confirmed by standard methods (Feng, 1996). Morandi *et al.* (2007) compared two methods for SEs

detection, mPCR technique and SET-RPLA but they did not found a good correlation between them. We have also compared two techniques and, according to them, the results obtained with SET-RPLA and LC-ESI/MS-MS are similar but not the same. SEB present in one sample have been only detected by spectrometric technique. This finding may be explained by the fact of the different sensibility between the techniques used.

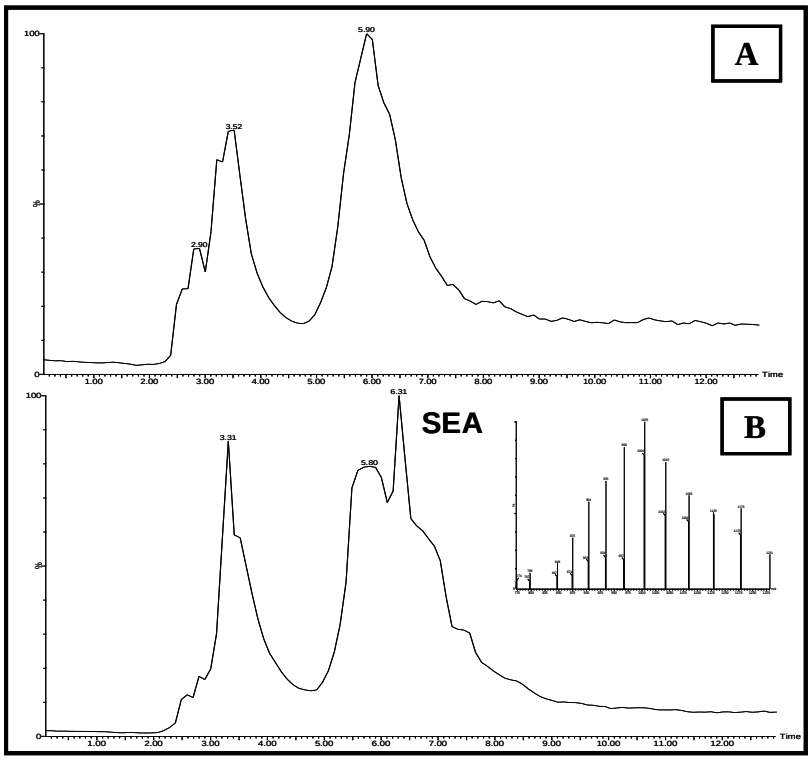

Figure 17. LC–MS chromatograms; (A) bacteria culture non-contaminated, (B) bacteria culture contaminated by SEA and mass spectrum of the peak obtained.

One of the most important advantages of LC-ESI/MS is that this method allows a quantification of the enterotoxins detected. SET-RPLA is a semiquantitative method; some works tried to

calculate the recovery of toxin from foods by the concentration of toxin detected by the RPLA method and the volume of the supernatant fluid (Park and Szabo, 1986). However, the concentration of toxin by this method is calculated discontinuously by the toxin titer of a sample and the sensitivity of toxin. For this reason, it is thought impossible to evaluate the exact recovery of toxin from a sample by RPLA.

5.7. Conclusions

The microbial quality of kitchen surfaces, with regard to the presence of TSST-1 is good. Despite this, extreme hygiene practices should be exercise in the surfaces of contact with food, as well as the hands of the manipulators.

An extraction procedure based on a mixture of dichloromethane and acidified water, followed by SDS-PAGE then detection with MALDI–TOF mass spectrometry can be successfully applied to the analysis of the enterotoxin most frequently implicated in foodborne diseases (SEA) from milk. The peptide sequence coverage of this compound shows that under the proposed conditions, most of the more intense peaks appearing in the mass spectra can be assigned to peptide fragments of the digested protein.

HPLC-DAD could be applied to bacteria culture isolates from food samples for the detection of SEB. The proposed technique allows a rapid identification and quantification of the whole

protein based on its retention time and UV spectra compared with a standard to avoid the possibility of obtaining false positives.

LC-ESI/MS method proposed allows multiple analytes detection in a single analysis, true identification based on the obtained spectra and the possibility of quantifying them at the same time. The quality of the data achieved would be much more and faster than the classical techniques. The method is a good tool for analysis of both exotoxins that are the most prevalent compounds in staphylococcal food poisoning worldwide; this technique is less time-consuming. The use of a sensitive and moderately time consuming method to detect SEA and SEB may be an advantage in microbial quality assurance of food products.

LC-ESI/MS method can be also applied to detect and quantify SEs from bacteria cultures. These advantages should lead to better control and a subsequent reduction of staphylococcal food poisoning outbreaks.

CONCLUSIONS

The information compiled from the literature review and results obtained made possible to establish the following conclusions:

1. Percentage or polar compounds present at frying oils used at university restaurants has decreased in last four years. Implementation of a Hazard Analysis and Critical Control Point (HACCP) plan along with routine inspections for monitoring the quality of the frying oils are important tools to guarantee the food safety.

2. The study reflects the absence *Anisakis simplex* parasite in studied fish samples at university restaurants. Implementation of the Spanish R. D. 1420/2006 on prevention of parasitosis by *Anisakis* in fish samples guarantees food safety. Fish served at university restaurants are bought frozen, are frozen at restaurants (at -20 C for 24 h) or cooked for at least 1 min at least 60 ºC, to guarantee the destruction of parasite larvae.

3. Microbiological analysis of cooked meals served at foodservice establishments al Universitat de València has revealed that,
   - Attending to mesophilic aerobic counts, vegetables are the most contaminated products being mainly broccoli (50%) and eggplant (40%) while egg- (hardboiled egg; 33%), fish- (whitefish from fresh water; 30%) and cereals-based (rice; 27%)

dishes are the following products with high percentage of unacceptable microbial quality.

- Attending to Enterobacteriaceae microbial quality, orange juice samples obtained from metal jug (81%) are the most contaminated, followed by vegetable- (broccoli; 70%) and egg-based (hardboiled egg; 60%) dishes.
- *S. aureus* was found in vegetable- (lettuce, French beans and potato in a range from 1.2 to 8%), meat-based (pig, beef and poultry in a range from 0.5 to 2.5%) dishes and orange juice (1% of the analyzed lots).
- *E. coli* was detected in vegetable- (lettuce; 6.6%) and meat-based (pig and poultry in a range from 1 to 1.25%) dishes.
- *Salmonella* spp. was positive in vegetable-based (lettuce; 0.7%) dishes and orange juice (1%) sampled from metal jug.
- *L. monocytogenes* was not detected in any studied samples.
- Storage of beverages in metal jugs for a long time has evidence to contribute with a high level of microbial contamination.
- Food products which need a manipulation after heat treatment present the highest level of contamination.

4. The presence of studied microorganisms in food samples, as the incidence of *S. aureus,* which indicates cross-contamination, evidence that several handling practices require more attention. Fruits and vegetables cleaning, preservation processes and

maintenance of adequate temperature of final products can be established as a critical control points.

Our results emphasize the importance of strict hygiene during handling practices in order to avoid contamination of the final food product. Correct handling of food products as well as an efficient cleaning and sanitization of their containers recipients are imperative to maintain the quality and safety of meals served at restaurants.

5. Form the study of *E. coli* heat labile toxin, it could be concluded that,
   - The B subunit of *E. coli* heat labile enterotoxin can be primarily characterized by a molecular mass determination by MALDI-TOF-MS and LC/ESI MS. The presence of one intramolecular disulfide bridge in the B-chain protein is readily verified by IAA reduction and MALDI-TOF MS.
   - Even so LT production by *E. coli* can drastically vary among ETEC strains and depending of culture conditions, tryptic soy broth has shown to be the most efficient culture medium for *E. coli* growth and enterotoxin production.
   - Although if several food samples from University restaurants were contaminated by *E. coli* strains, none of them showed to be contaminated by LT producer strains.

6. The analysis of kitchen surfaces, with regard to the presence of *S. aureus* and TSST-1 evidence that,

- 5.8% of total studied surfaces were contaminated by *S. aureus*, being mainly in dish towels followed by workers' hands, cutting boards, stainless steel tables, and slicers. For this reason, efficient cleaning and sanitization practices of food contact surfaces are imperative to maintain the quality of foods served at university restaurants.
- About the presence of TSST-1 toxin, establishment surfaces are free of risks. Despite this, TSST-1 producing *S. aureus* strain has been detected at first time in Spain, in a worker hand. This fact indicates that continued hygiene practices should be exercise in the surfaces of contact with food.

7. Regarding to methods proposed for staphylococcal enterotoxins identification it can be concluded that,
   - An extraction procedure based on a mixture of dichloromethane and acidified water, followed by SDS-PAGE then detection with MALDI-TOF mass spectrometry can be successfully applied to the analysis of the staphylococcal enterotoxin A from milk. The peptide sequence coverage of this compound shows that under the proposed conditions, most of the more intense peaks appearing in the mass spectrum can be assigned to peptide fragments of the digested protein.
   - HPLC-DAD could be applied to bacteria culture isolates from food samples for the detection of SEB. The proposed technique allows a rapid identification and quantification of

the whole protein based on its retention time and UV spectrum.

- LC-ESI/MS method proposed allows multiple analytes detection in a single analysis. This technique is quickly and selectively and is a good tool for analysis of both exotoxins that are the most prevalent compounds in staphylococcal food poisoning worldwide.

8. The study of the presence of enterotoxigenic *S. aureus* in food samples collected from university restaurants evidence that 7% of examined samples were contaminated by strains which demonstrated to be enterotoxigenic. All *S. aureus* isolates produced SEA and only 50% SEB. LC-ESI/MS method can be applied to detect and quantify SEs from bacteria cultures and comparing with SET-RPLA technique, the proposed method showed more accuracy, lower limit of detection and possibility of quantification.

CONCLUSIONES

La revisión bibliográfica efectuada y los resultados obtenidos permiten establecer las siguientes conclusiones:

1. El porcentaje de compuestos polares presentes en los aceites de fritura utilizados en los servicios de restauración universitarios ha disminuido durante los últimos cuatro años. La implantación de sistemas de Análisis de Peligros y Puntos Críticos de Control (APPCC) junto con inspecciones rutinarias para monitorizar el estado de los aceites de fritura, representan importantes herramientas para garantizar la calidad y seguridad alimentaria.

2. El estudio realizado muestra la ausencia del parásito *Anisakis simplex* en las muestras de pescado tomadas de restaurantes universitarios. El cumplimiento del Real Decreto 1420/2006 para la prevención de parasitosis causadas por *Anisakis* en pescado, contribuye a garantizar la seguridad alimentaria de los platos a base de pescado. De acuerdo con la legislación vigente, los restaurantes universitarios compran el pescado congelado, lo congelan en los servicios de restauración (a -20 C durante 24 h) o es sometido a procesos de cocción donde se alcanzan 60 ºC durante al menos 1 minuto, para asegurara la destrucción de las larvas de *Anisakis*.

3. El análisis microbiológico de los menús servidos en los servicios de restauración de la Universitat de València mostró que:

- Atendiendo a la presencia de aerobios mesófilos totales, los vegetables fueron los productos más contaminados, destacando entre ellos el brócoli (50%) y las berenjenas (40%), mientras que los huevos (huevos hervidos; 33%), los platos de pescado (pescado blanco de agua dulce; 30%) y los platos a base de cereales (arroz; 27%) fueron, después de las verduras, los productos que presentaron mayor porcentaje de muestras con calidad microbiológica inaceptable.
- Respecto a la presencia de Enterobacteriaceas, las muestras de zumo de naranja procedentes de jarras metálicas (81%) fueron las más contaminadas, seguidas por los platos a base de vegetales (brócoli; 70%) y huevos (huevos duros; 60%).
- *S. aureus* fue aislado en muestras de vegetales (lechuga, judías verdes y patatas en un rango de 1.2 a 8%), carnes (cerdo, ternera y pollo en un rango de 0.5 a 2.5%) y zumo de naranja (1% de los lotes analizados).
- Se detectó la presencia de *E. coli* en muestras de vegetales (lechuga; 6.6%) y carne (cerdo y pollo en un rango de 1 a 1.25%).
- Se aisló *Salmonella* spp. en platos a base de vegetales (lechuga; 0.7%) y en muestras de zumo de naranja (1%) tomadas de zumos conservados en jarras de metal.
- No se detectó presencia de *L. monocytogenes* en ninguna de las muestras analizadas.
- El almacenamiento y conservación de algunos tipos de bebidas durante largos períodos de tiempo, en jarras de acero

ha demostrado ser un punto crítico que contribuye al aumento de los niveles de contaminación del alimento.

- Aquellos alimentos que necesitan una manipulación por parte del personal del servicio de restauración, tras sufrir un tratamiento térmico, son más susceptibles de presentar elevados niveles de contaminación.

4. La presencia de algunos de los microorganismos analizados en las muestras de alimentos, como puede ser la incidencia de *S. aureus*, indicador de contaminación cruzada, pone en evidencia que diversas prácticas de manipulación requieren mayor cuidado y atención. El lavado de frutas y verduras, así como los procesos de almacenamiento, conservación y mantenimiento de la temperatura de los alimentos ya cocinados pueden establecerse como puntos críticos de control.

   Los resultados obtenidos resaltan la importancia de una higiene estricta durante la manipulación de alimentos con la finalidad de eliminar o reducir la contaminación del producto final. Unas buenas prácticas de manipulación, así como una limpieza correcta y eficiente de los recipientes en contacto con los alimentos son necesarios para mantener la calidad y la seguridad de los menús servidos en los restaurantes.

5. Del estudio realizado sobre la toxina termo lábil de *E. coli*, se puede concluir que:

- La subunidad B de la toxina termolábil de *E. coli* puede ser caracterizada mediante la determinación de su masa molecular por MALDI-TOF-EM y CL/ESI EM. La presencia de un puente disulfuro intramolecular en la subunidad B se ha verificado por reducción con IAA y análisis por MALDI-TOF EM.
- Pese a la gran variabilidad existente en la producción de toxina termolábil por *E. coli,* entre las diferentes cepas enterotoxigénicas, el caldo de Tryptic soy broth ha demostrado ser el medio más adecuado para el crecimiento de *E. coli* y para favorecer la producción de toxina.
- Aunque diversas muestras de alimentos de los restaurantes de la Universitat de València presentaron contaminación por *E. coli*, ninguna de las cepas aisladas demostró ser productora de LT.

6. El análisis de superficies en contacto con alimentos en cocinas, respecto a la presencia de *S. aureus* y toxina TSST-1 evidenció que,

- El 5.8% del total de las superficies estudiadas estaban contaminadas por *S. aureus.* Las muestras que presentaron niveles mayores de contaminación fueron los trapos de cocina, seguidos de las manos de los trabajadores, las tablas para cortar alimentos, las mesas de trabajo y las cortadoras de fiambre. Por este motivo, la práctica de una limpieza y desinfección continuada y eficiente de las superficies en contacto con los alimentos son necesarias.

- En lo relativo a la presencia de la toxina TSST-1, las superficies de trabajo están libres de riesgo. Sin embargo, se aisló, por primera vez en España, una cepa de S. *aureus* productora de toxina TSST-1 en las manos de un manipulador de alimentos. Este hecho indica que es necesaria una higiene continua de las manos de los manipuladores.

7. En cuanto a los métodos propuestos para la identificación de toxinas estafilocócicas se puede concluir que:
   - El método de extracción basado en una mezcla de diclorometano y agua acidificada, seguida por SDS-PAGE y la detección mediante espectrometría de masas por MALDI-TOF puede ser aplicada satisfactoriamente para el análisis de enterotoxina A en leche. El reconocimiento de la secuencia peptídica de SEA demuestra que, con las condiciones propuestas, la mayoría de los picos de mayor intensidad que aparecen en el espectro de masas pueden ser asignados a fragmentos obtenidos de la digestión tríptica de la proteína.
   - El sistema de detección de SEB mediante CL-DAD puede ser aplicado a cultivos de bacterias aisladas de muestras de alimentos para la detección de la enterotoxina. La técnica propuesta permite una rápida identificación y cuantificación de la proteína entera basada en el tiempo de retención y el espectro uv.
   - El método de análisis por CL-ESI/EM propuesto permite la identificación y cuantificación simultánea de varias exotoxinas

de manera rápida y selectiva, incluidas las dos más frecuentes en todo el mundo productoras de intoxicación alimentaria por estafilococos.

8. El estudio de la presencia de *S. aureus* enterotoxigénico en muestras de alimentos procedentes de servicios de restauración mostró que un 7% de los alimentos analizados estaban contaminados por cepas enterotoxigénicas. Todas las cepas de *S. aureus* aisladas produjeron SEA y, solo un 50% produjo SEB. El método de análisis de SEA y SEB por CL-ESI/EM puede ser aplicado satisfactoriamente para detectar y cuantificar enterotoxinas en cultivos bacterianos, ventajosamente frente a la técnica de SET-RPLA, debido a su mayor selectividad, menor límite de detección y posibilidad de cuantificación.

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