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REVIEW

MYCOTOXIN ANALYSIS, MYCOTOXIN-PRODUCING FUNGI ASSAYS AND MYCOTOXIN TOXICITY BIOASSAYS IN FOOD MYCOTOXIN MONITORING AND SURVEILLANCE

APPROCCIO OLISTICO AL MONITORAGGIO E SORVEGLIANZA DELLE MICOTOSSINE: ANALISI DELLE MICOTOSSINE, DEI FUNGHI MICOTOSSIGENICI E BIOASSAYS

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ABSTRACT

Due to the high economic and health impact of mycotoxins on feed/food safety and animal/human health, the EU has published regulations on undesirable substances in feedstuffs and foodstuffs and the requirements for regular testing. In this context, there is an increasing need for rapid analysis methods for mycotoxins, to support the official methods. An overview of emerging rapid methods for a ho-

RIASSUNTO

La contaminazione da micotossine sia degli alimenti destinati al consumo umano che di quelli destinati agli animali rappresenta un aspetto legato alla sicurezza alimentare di notevole impatto sanitario ed economico. Con la pubblicazione ed i continui aggiornamenti dei regolamenti relativi alla presenza di sostanze indesiderabili negli alimenti, l'UE riconosce prioritaria la problematica della sicurezza alimentare e l'attività di con-

- Key words: bioassay, diagnostic tools, food safety, mycotoxins, toxigenic fungi -

listic approach to mycotoxin monitoring and surveillance is presented. This paper focuses on recent developments in technologies in mycotoxin analysis, detection of mycotoxin-producing fungi and mycotoxin toxicity cell-based bioassays as rapid tools for evaluating food and feed mycotoxin contamination. Applications and limitations in accordance with legislation requirements and the complementary aspects of different technological approaches are discussed.

trollo degli alimenti, compresi quelli destinati al consumo animale. In tale contesto, la necessità di metodi diagnostici rapidi ed adeguatamente validati per la determinazione della contaminazione da micotossine, in supporto ai metodi ufficiali di analisi, diventa sempre più urgente. In questa review vengono pertanto descritti metodi rapidi emergenti volti alla valutazione della contaminazione con micotossine, con funghi micotossigenici e bioassay basati sull'impiego di colture cellulari per la valutazione della cito-tossicità delle micotossine. Vengono inoltre discussi aspetti relativi all'applicazione, limiti e complementarietà dei metodi in relazione agli obiettivi specifici delle diverse analisi nonché alle richieste normative specifiche.

INTRODUCTION

Mycotoxins are worldwide contaminants of feed and food. Episodes of mycotoxin contamination have been reported in both developing and developed countries. It has been estimated that up to 25% of the world's crops grown for feed and food may be contaminated with mycotoxins (SMITH *et al.*, 1994; FINK-GREMMELS, 1999; HUSSEIN and BRASEL, 2001). Feed contamination can be a hazard for the safety of food of animal origin and can contribute to mycotoxin intake by the human population (JORGENSEN and PETTERSEN, 2002; PETZINGER and WEIDENBACH, 2002; MONACI and PALMISANO, 2004; PIETRI *et al.*, 2006). While it is impossible to fully eliminate the presence of undesirable substances, it is important to monitor and then reduce their content in feed and food. The knowledge and control of mycotoxin contamination and distribution is a worldwide objective of producers, manufacturers, regulatory agencies and researchers due to the high economic and sanitary impact on food safety and animal/human health. Since commodity trade and globalisation will increase the risk to consumers due to contaminated feed and food, precautionary measures must be taken and products must be checked. The complex problem of safety and quality monitoring of commodities and processed food and the requirements of the feed and food industry and the frequent checks required by the international authorities necessitate surveillance and rapid diagnostic methods for detecting mycotoxin contamination.

Rapid methods are needed to determine the effectiveness of food safety measures, to determine legal compliance and to achieve logistical and operational goals. They would keep commodities and products moving rapidly through the marketing channels, save time, reduce costly investments in complex instruments and would require less technical training. Most rapid methods provide qualitative or semi-quantitative re-

sults and are recommended for use in sample screening. Methods are usually referred to as rapid when it takes only minutes to get a result. For mycotoxin assessment, speed is not the only factor to be considered. Other parameters are also fundamental, such as the skill level required to perform the assay, reliability, non-destructivity, yes/no or semi-quantitative response, multi-mycotoxin analysis in a single test and cost of analysis. Currently, there are three main factors considered when developing rapid methods for mycotoxin analysis to reduce the quantity of assays and, therefore, shorten time and lower costs for feed and food quality control: 1) improvement of speed, user-friendliness, reliability and non-destructiveness, 2) use in a non-laboratory environment and 3) simultaneous determination of multiple mycotoxins (MARAGOS, 2004).

The development of low-tech, rapid and inexpensive methods for mycotoxin surveillance is a world health imperative and several novel approaches are being developed. It is obvious that the detection of mycotoxins themselves must be distinguished from the detection of mycotoxin-producing fungi and evaluation of mycotoxin toxicity. Although the latter assays are not direct mycotoxin detection methods, there is emerging evidence that they can be used to predict mycotoxin contamination levels. They could play a complementary role in mycotoxin monitoring and surveillance. It must also be noted that selecting the appropriate analysis method depends on the intended use of the method (*fit for purpose analysis*) and the purpose of the analysis: screening, monitoring, control and/or exposure studies (MIRAGLIA *et al.*, 2005).

This overview presents emerging and very promising rapid analytical approaches for feed/food safety assessment regarding mycotoxins, mould contamination and mycotoxin toxicity. The purposes, possibilities, limitations and complementary aspects of different technological approaches to mycotoxin surveillance are discussed.

MYCOTOXIN DETECTION: RAPID CHEMICAL METHODS

Emerging technologies and their potential application in rapid mycotoxin detection have been reviewed (MARAGOS, 2004; PITTET, 2005, KRSKA and WELZIG, 2006; ZENG *et al.*, 2006; GORYACHEVA *et al.*, 2007). The challenge of the emerging technologies is to demonstrate advantages over the more conventional and validated methods in order to satisfy different emerging demands in the field of mycotoxin surveillance such as rapid screening and sufficiently high sensitivity, multi-mycotoxin determination, applicability outside a laboratory and cost efficiency.

The best known rapid screening methods for mycotoxin detection, especially for screening raw materials, are antibody-based methods. The most common of these is the microtiter plate enzyme-linked immunoabsorbent assay (ELISA). The ELISA methods have been commercially available for many years and are extensively used as rapid screening methods. Kits are available in quantitative, semi-quantitative or qualitative formats. These methods are easy to use, fast and suitable for testing mycotoxins in the field. A review of the current status of commercially available ELISA methods and a critical evaluation of the performance characteristics has been presented by ZENG *et al.* (2006).

A list of commercial immunological test kits (and specifications) for the analysis of mycotoxins has been compiled by the European Mycotoxin Awareness Network (EMAN, www.mycotoxins.org). Some commercial kits have been successfully evaluated (performance tested) by the AOAC International Research Institute; some have AOAC International

Official Methods status or are in a collaborative study process to obtain such a status (SCHNEIDER *et al.*, 2004). These methods can be very rapid, with an assay time (time needed to detect mycotoxins in a single, pre-ground sample after extraction) that may be less than 30 min.

Within the concept of flexible *out of laboratory* testing, non-instrumental (visual) membrane based immunoassays (dipstick, lateral flow and flow-through tests) have been developed and are commercially available for several mycotoxins and matrices. Within minutes they provide either a yes/no determination of the presence of a target mycotoxin or a threshold (semi-quantitative) result. Advantages and disadvantages of these non-instrumental methods have been identified and discussed by several authors (PITTET, 2005; ZENG *et al.*, 2006; GORYACHEVA *et al.*, 2007). They note that the main advantages of non-instrumental ELISA methods are their field portability, they do not require any specialized equipment, and sample preparation is simple. The main disadvantages are that the interpretations are subjective, they are less sensitive and the cost/test ratio is higher than that with instrumental ELISA methods.

Although immunochemical methods have become one of the most useful tools for rapid mycotoxin monitoring and screening, the trade-off for simplification is usually lower sensitivity. The main problems with antibody-based methods are related to the characteristics of the antibody, test specificity (cross-reactivity), matrix interference and interpretation of the results, if the method is semi-quantitative, when the mycotoxin concentration is close to the method cut-off level. Since validation studies have not been made for the ELISA methods for all commodities, they can only be used for those matrices for which they have been validated.

Apart from ELISA, the more recent and best candidates for mycotoxin analytical methods are capillary electrophoresis (CE), fluorescence polarization immunoassay (FPI) and surface plasmon resonance (SPR). CE methods are laboratory-based methods because of the size and required automation of the instrumentation. The FPI and SPR methods are much more portable and can be used outside the laboratory and they have reached the stage of commercialization.

In capillary electrophoresis the mycotoxins are separated from matrix components in a narrow capillary, with increased speed and efficiency of analysis. After separation, analytes are detected using fluorenscence or UV absorbance. To attain the sensitivity needed for the analysis of mycotoxins in food, fluorescence detection is required. However with sufficient clean-up, even mycotoxins without a native fluorescence can be labelled and detected at relevant levels. CE methods for aflatoxins, fumonisins, ochratoxin A (OTA), deoxynivalenol (DON), moniliformin (MON), and zearalenone (ZEN) have been reviewed by MARAGOS (1998).

Capillary electrophoresis with laser-induced fluorescence (CE-LIF) was used to quantify OTA in roasted coffee, maize and sorghum with a sensitivity comparable to that of liquid chromatography (CORNELI and MARAGOS, 1998). Recent advances in CE-LIF for mycotoxin analysis include the use of cyclodextrins to increase the sensitivity of the assay and improve the detection limit. MARAGOS and APPEL (2007) tested a wide variety of cyclodextrins in an attempt to develop a CE-LIF method with high sensitivity for ZEN (range from 5 ng/g to 500 ng/g).

A combination of CE with immunoassay has been used to analyse fumonisin B1 in maize (MARAGOS, 1997). In this assay, an antibody is combined with sample extract and a fluorescently labelled fumonisin (tracer). Unbound and bound tracers are separated in an electrical field. If fumonisin is present in the sample, the signal of the unbound

tracer increases and the signal of the bound tracer decreases, indicating the presence of the toxin. The sensitivity of CE immunoassay can be very high depending on the antibody concentration and affinity for the mycotoxin under strong electric field conditions. The main advantage of CE is the possibility of reaching a sensitivity comparable to that of established high performance liquid chromatography (HPLC) methods and the absence of an organic solvent during the determination step. CE is an inexpensive, sensitive, and non-polluting technology. A combination of CE with immunoassay allows a simultaneous multi-component analysis due to the high resolving power of CE. Rapid detection of multiple mycotoxins is of crucial interest in the food control area. Up to now, rising CE immunoassay for simultaneous and multi-analyte determination has only been reported for drugs (GORYACHEVA *et al.*, 2007).

FPI are solution-based assays in which a mycotoxin-fluorophore conjugate (tracer) is used. The analytical signal is the fluorescence polarization value of the label, which corresponds to its rotation rate in solution. The addition of antitoxin antibody results in the formation of an immune complex of the tracer with the antibody that has a lower rotation rate and, consequently, a higher fluorescence polarization value. Applications of FPI assays have been described for the detection of DON, fumonisins, aflatoxins, ZEN and OTA in cereals, semolina and pasta (MARAGOS, 2004; GORYACHEVA *et al.*, 2007). Good correlations have been found between comparative analyses performed by FPI and HPLC. The main advantages of FPI are high sensitivity and low matrix interference. The challenge with FPI development will be to discover the best combination of antibody and tracer to ensure adequate sensitivity and reproducibility. The potential speed of FPI assays combined with the portability of commercially available devices, makes this a promising technology for mycotoxin detection. One limitation of FPI is that it cannot be used to simultaneously detect several individual analytes in a single assay (tube). Since the analytical signal in FPI is the polarisation value of a solution, the signal is related to one or more compounds without discrimination. However FPI may potentially be used as an array multi-immunoassay with separate tubes for parallel (simultaneous) detection of several analytes (GORYACHEVA *et al.*, 2007).

Applications of SPR assay to detect DON, fumonisins, aflatoxins, ZEN and OTA have been developed and optimized (DALY *et al.*, 2000; SCHNERR *et al.*, 2002a; TUDOS *et al.*, 2003; VAN DER GAAG *et al.*, 2003). SPR is a measure of mass changes that occur in a sensor surface. The changes in mass concentration are due to the binding and dissociation of interacting molecular complexes (mycotoxins bind to specific antibodies) to the thin metal film of the biosensor surface. SPR is more sensitive to aflatoxin B1 than ELISA assay. Studies on naturally contaminated samples showed that SPR results are in agreement with liquid chromatography-mass spectrometry (LC-MS) measurements (TUDOS *et al.*, 2003; VAN DER GAAG *et al.*, 2003). A technique for simultaneously detecting four different mycotoxins in a single measurement using commercially available portable SPR equipment has been recently reported (VAN DER GAAG *et al.*, 2003).

The aforementioned technologies based on the use of immunoassay for mycotoxin screening are at various stages of development as useful rapid analytical tools. Some are advanced enough for field studies and are already commercially available, some are at a transition phase between research and application to the analysis of food/feed samples, and others still have to be validated by several laboratories. The main goals for research and application of immu-

noassay for mycotoxins are sensitivity improvement, validation for each commodity, matrix effect reduction, simplification and shorter analysis time and development of simultaneous multiple mycotoxin determination tests. The few rapid immunochemical multi-mycotoxin methods were reviewed by GORYACHEVA *et al.* (2007).

In an emerging challenge to find sensors for mycotoxins, non-biologically based binding, such as molecularly imprinted polymers (MIPs), has been developed (MARAGOS, 2004; LOGRIECO *et al.*, 2005; KRSKA *et al.*, 2005). Rapid application of MIPs is expected if affinity problems can be overcome. Mimicking antibodies is the basic idea of MIP technology. MIPs are generated by the polymerization of a functional monomer in the presence of cross-linker, radical initiator and template (analyte) molecules. After polymerisation, the removal of the template produces cavities with specific binding sites for the template molecules. Potential applications of MIPs in mycotoxin analysis are foreseen at the level of solid phase extraction, biosensor device design and preparation of chromatographic matrices for separations. A functional polymer material with high affinity for OTA has been made and used to prepare micro-columns for selective solid-phase extraction (ZHOU *et al.* 2004; ZHOU and LAI, 2004). The detection limit was 5.0 mg/L, whereas the mean recovery was 103±3% for MIP. These results show the excellent affinity strength of the MIP to bind OTA during extraction. Recovery, using the control polymer micro-column (NIP), was 125±5% due to poor selectivity of the control polymer particles.

The preliminary results of MIPtechnology in ZEA, DON and OTA analysis have been reported (VISCONTI and DE GIROLAMO, 2005; KRSKA and WELZIG, 2006). Although the affinity of the MIPs is not yet competitive with that of antibodies, this technique offers good potential for further development.

MYCOTOXIN-PRODUCING FUNGI: RAPID METHODS

The detection and control of mycotoxigenic fungi is crucial for preventing toxins from entering the food chain and is critical for disease management. Rapid detection technologies to assess the presence of mould can be divided into two main classes: phenotypic, which rely on expressed characteristics, and genotypic, which evaluate the presence of unique genetic characteristics.

The presence and growth of fungi cause physical and chemical changes in the commodities which can then be used to identify fungally-infected material. The most promising methods, in terms of rapidity, sensitivity for early detection, time consumption and good correlation with mycotoxin contamination, are infrared spectroscopy and the electronic nose.

Infrared (IR) spectroscopy has been evolving continuously, as can be deduced by comparing the old mid-IR equipment manufactured in the 1950s that was based on dispersive monochromators with the present customized near infrared (NIR) instrumentation. The incorporation of the Fourier transform technique (FT), together with the interferometric spectrometers into the mid-IR instruments, has increased the use of this technique in food analysis (IBANEZ and CIFUENTES, 2001). Although NIR spectroscopy has been used routinely for many years as a rapid method in the feed and food industry for determining constituents, such as humidity, proteins and lipids with a precision that is comparable to that of the official methods of analysis (CEN and HE, 2007), only a limited number of publications have been concerned with mycotoxins and NIR spectroscopy. This is because the concentration of mycotoxins normally found in feed and food has been considered to be too low for this technique. Some infrared spectroscopic techniques

were first used in single kernel analysis to detect fungal infection in corn (DOWELL *et al.*, 1999). However, it is difficult to draw conclusions about a whole sample based on results from investigations of single kernels. NIR and mid-infrared (MI) spectroscopy with attenuated total reflection (IR/ATR and FT-IR/ATR) have been used to rapidly detect fungal infection and estimate fungal metabolites and mycotoxins in naturally- and artificially-contaminated products (KOS *et al.*, 2002, 2003; PETTERSON and ABERG, 2003; BERARDO *et al.*, 2005). The results of multivariate analysis to extract additional information from the recorded spectra showed that these techniques can be used as tools and models for detecting moulds, but also for predicting the presence of mycotoxins (Fig. 1). Chemometric models applied to FT-IR/ATR analysis provided a correct classification of non-contaminated and contaminated maize with DON (KOS *et al.*, 2003). The method developed enabled samples with DON content as low as 310 μg/kg to be separated from clean samples, and the percentage of correctly classified samples even reached 100%. The maximum level and guidance value proposed by the EU for maize intended for human and animal consumption are 1,750 μg/kg and 8 mg/kg, respectively (Commission Regulation (EC) N. 1126/2007; Commission Recommendation 2006/576/EC). The classification performance of FT-IR/ATR analysis can be improved by optimising the sample preparation procedure and applying particle size analysis to samples (KOS *et al.*, 2007). Repeatability and classification for DON are better in sieved samples than in non-sieved samples. Moreover, the DON level analyzed in the maize fraction is a good estimate of the overall toxin content. An attempt was made to quantify the DON content by applying a PLSr (Partial Least Square regression) model to the data. A good correlation was found between the spectral data and reference concentrations for DON (KOS *et al.*, 2003). FT-IR/ATR spectroscopy has been used to detect ochratoxin A in dried vine fruit (GALVIS-SANCHEZ *et al.*, 2007). Samples with OTA levels higher than 20 μg/kg were separated from samples that were contaminated with lower levels of OTA or were not contaminated. NIR spectroscopy has been used to determine DON in wheat and fumonisin B1 in maize (PETTERSON and ABERG, 2003; BERARDO *et al.*, 2005). Using NIR, it is possible to predict DON concentration in wheat kernels at levels higher than ca. 400 μg/kg (PETTERSON and ABERG, 2003). These results indicate that IR spectroscopy can be used to ac-

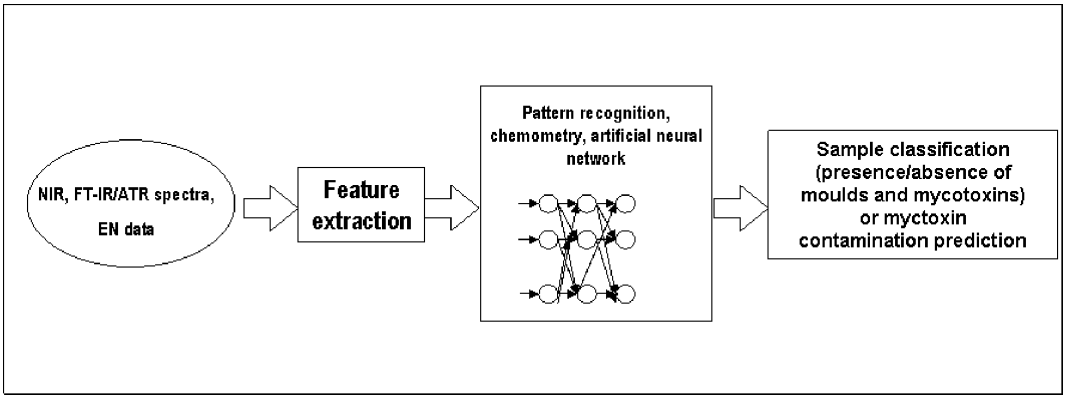

Fig. 1 - Application of infrared (IR) and electronic nose (EN) technologies to mycotoxin surveillance.

curately predict the presence or absence of mycotoxins in cereals.

The development of the electronic nose (EN) technology has stimulated interest in the use of volatiles and odours as indicators of food quality. Fungal spoilage induces nutritional losses, off-flavours, organoleptic deterioration and formation of mycotoxins. Research studies have correlated fungal activity with the production of volatile metabolites that have been characterized by gas chromatography-mass spectrometry (for review see MAGAN and EVANS, 2000). These authors conclude that the accumulation and pattern of fungal volatiles can be used as indicators of fungal activity and as taxonomic markers to differentiate between fungal species and between toxigenic and non-toxigenic fungal strains. Since volatile headspace analysis can be evaluated as a whole by using an electronic nose, this technique is becoming widespread to evaluate mould spoilage and quality and safety of food and feed. An EN is an instrument which comprises an array of electronic chemical sensors with partial specificity and an appropriate pattern recognition system, that is capable of recognizing simple or complex odours (GARDNER and BARTLETT, 1994). Sensor array formats interact with different volatile molecules and provide an electronic signal that can be utilised effectively as a fingerprint of the volatile pattern associated with the product. The electronic nose does not distinguish each volatile substance, but expresses the global odour of a product. With the aid of appropriate mathematical and statistical methods, the EN may recognize the odour pattern from a sample and distinguish it from other samples (GARDNER and BARTLETT, 1994). The use of an EN to evaluate the quality of stored grain has been reported. Sensor technology can determine the mycological quality of grains. EN technology was first used to differentiate between non-infected and infected samples with different species or strains of fungi, through variations in the metabolic pathway due to the contamination of grains. The ability of an EN to differentiate grains and bakery products, clean or contaminated (naturally or infected) with different mould species has been demonstrated (KESHRI *et al.*, 1998; BORJESSON *et al.*, 1993; MAGAN and EVANS, 2000; OLSSON *et al.*, 2000; VINAIXA *et al.*, 2004; BALASUBRAMANIAN *et al.*, 2007; PAOLESSE *et al.*, 2006). Detection and differentiation between mycotoxigenic and non-mycotoxigenic strains of *Fusarium* spp. using volatile production profiles evaluated by an EN has been reported (KESHRI and MAGAN, 2000; MAGAN and EVANS, 2000; FALASCONI *et al.*, 2005; PRESICCE *et al.*, 2006; SAHGAL *et al.*, 2007). Further studies carried out with EN technology have been made to evaluate the possibility of using fungal volatile metabolites as indicators of the presence of mycotoxins. With this aim, multivariate analysis has been carried out to extract additional information from olfactometric data. The results show that these techniques are promising tools and models for evaluating the presence of mycotoxins (Fig. 1). Results from a study carried out on naturally-contaminated barley samples showed that it was possible to use volatile compounds to predict whether the OTA level in samples was above or below 5 μg/kg; 7 of 37 samples were misclassified (OLSSON *et al.*, 2002). EN analysis provided correct classification of naturally-contaminated maize with aflatoxins. When multivariate classification (cross validated Linear Discriminant Analysis - LDA) was applied to EN data from 28 maize samples split into two classes (9 samples non-contaminated *vs* 19 contaminated), the model correctly classified all of the samples (CHELI *et al.*, 2007). EN analysis and cross validated LDA were also applied to wheat in the case of DON contamination. When data from 41 samples were split into three classes of samples (non-contaminated, contaminated below or above

the legislation limit - 1,750 μg/kg), only one sample contaminated below the legislative limit was incorrectly classified. Quantification was also attempted in order to predict DON levels in barley and wheat using EN analysis (OLSSON *et al.*, 2002; TOGNON *et al.*, 2005; DELL'ORTO *et al.*, 2007). A positive correlation was found between the electronic nose data and the reference concentration of DON. However the performance of the prediction model was quite low (PRESS 0.65, R^2 0.63, adjR^2 0.63) (TOGNON *et al.*, 2005; DELL'ORTO *et al.*, 2007). Apart from cereals, another example of applying the head space sensor array system is the detection of aflatoxin M1 in raw milk (BENEDETTI *et al.*, 2005).

The methods described (IR spectroscopy and EN), which enable high sample throughput with no or limited sample preparation (e.g. grinding), are powerful and rapid tools for monitoring and rapidly screening large samples of cereals to distinguish between mycotoxin-contaminated and clean lots at the levels proposed by the EU legislation. Commercial instruments are available on the market and their use in the feed/food industry became immediate. Current major restrictions are high matrix dependence, lack of appropriate calibration material, the need for increased sensitivity, repeatability and optimization of the analysis and development of suitable multivariate methods in order to develop models for correct mycotoxin classification and quantification.

The genotypic approach for the rapid identification and quantification of mycotoxigenic fungi is possible using several polymerase chain reaction (PCR)-based methods (random amplification of polymorphic DNA (RAPD), mitochondrial restriction fragment length polymorphisms (RFLP), real-time (RT)-PCR and competitive PCR. Advances in molecular diagnosis of mycotoxigenic fungi have been reviewed by EDWARDS *et al.* (2002), SEIFERT and LEVESQUE (2004) and MULÈ *et al.* (2005). It has been proved that diagnostic methods based on PCR are rapid, specific and highly sensitive. These methods can be a useful tool for early detection and traceability of the different species of mycotoxigenic fungi along the production chain. Furthermore, implementation of DNA chip technology may provide a rapid and easy-to-use tool for identifying several toxigenic fungi strains in one step. A DNA microarray based on multiplex single nucleotide polymorphism (SNP) analysis has been developed for the detection and identification of trichothecene- and moniliformin-producing *Fusarium* species (KRISTENSEN *et al.*, 2007). The authors postulated that the method is highly sensitive, fast and has the potential for (semi-)quantification. Genetic markers have been studied for mycotoxin-producing fungi as well as the mycotoxins themselves, in order to identify genes which may be related to mycotoxin synthesis (MAGAN, 2006). The quantification of *Fusarium* species in comparison with mycotoxin content has been studied by several authors. A positive, linear and highly significant (r^2 average 0.98) correlation between DNA of mycotoxin-producing *Fusarium* and DON concentrations in field-inoculated wheat and barley samples has been reported (NIESSEN and VOGEL, 1998; SCHNERR *et al*, 2002b; WAALWIJK *et al.*, 2004; LEISOVA *et al.*, 2006). Weaker correlations have been found between DON and trichothecene-producing *Fusarium* species in naturally infected grains (EDWARDS *et al.*, 2001). These findings indicate that the relationships between fungal biomass and DON in harvested grain may be affected by several factors, such as the presence of various strains of *Fusarium* spp that have different capabilities for the production of mycotoxins, host and/or environmental conditions. A PCR-based analytical approach has been used to quantify the presence of DON-producing *Fusarium* spp. in wheat grains and along the bread

production chain (TERZI *et al.*, 2007). A correlation between *Fusarium* DNA and DON concentration was confirmed in wholemeal, flour and bread. An association between trichothecene and *Fusarium* DNA has been reported in settled grain dust that may represent a possible health risk for grain farmers (HALSTENSEN *et al.*, 2006). A strong correlation between trichothecene HT-2 toxin or T-2 toxin and *tri5* (trichodiene synthase gene) and *Fusarium langsethiae*-specific DNA was found. A moderate correlation was found between DON and a combination of *F. culmorum* and *F. graminearum*, and no correlation was found between DON and *tri5.* Promising results of PCR-based assay to detect potential fumonisin-producing *Fusarium* strains have also been reported (SANCHEZ-RANGEL *et al.*, 2005; JURADO *et al.*, 2006). A strong association between fumonisin production and *FUM1* gene was found in samples that had fumonisin levels higher than 12.6 ppm. The main restriction of the PCR assay is that it does not always detect the presence of fungi in samples with a low mycotoxin content particularly in seed samples (JURADO *et al.*, 2006). It has been suggested that the limiting and critical factor could be the very small amount of sample used for PCR assays.

To conclude, the use of DNA markers is a good and informative analytical approach for developing accurate, rapid, specific and easy diagnostic protocols for detecting mycotoxin-producing fungi at very early stages of infection. Development of a PCR assay for critical toxigenic fungi or for genes involved in the biosynthesis of mycotoxins is promising. It can be used for a large-scale screening analysis of food or feed products to potentially reveal which particular mycotoxins may be present. Although positive, linear and highly significant correlations between DNA of mycotoxin-producing fungi and mycotoxins have been found, the use of fungi DNA markers as indicators for mycotoxin contamination should still be used with caution. In naturally-contaminated products, the effects of plant genotype, environmental conditions during growth and storage, genotype/environment interactions, presence of different fungi species with different mycotoxin-producing capacity and progression of fungal infection on mycotoxin content must be further investigated.

BIOASSAYS

Bioassays may also provide rapid, low-cost methods for detecting and monitoring mycotoxin contamination in feed and food. *In vitro* bioassay may be a complementary approach to the rapid methods described above. It can be used as a screening tool for mycotoxins based on their effects as well as an analytical tool in health risk studies to determine the response level above which adverse effects may be expected (Fig. 2). Bioassays, especially those using microorganisms and cell culture models, are prime candidates as they may be objective, fast and cheap large-scale screening tools for mycotoxin toxicity analysis. Besides providing a valuable screening tool for mycotoxin detection, bioassays have an additional value in that they can be used to evaluate and gain insight about the mechanisms of action of mycotoxins. When bioassays are used for mycotoxin surveillance analysis, the toxic effect can be determined with different methods. The parameters most commonly used to determine general cytotoxicity include the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide) test, and the evaluation of cell proliferation, DNA inhibition and protein synthesis.

Yeast-based bioassays have been developed to detect thricothecenes and zearalenone and other compounds with estrogenic activity (ENGLER *et al.*, 1999;

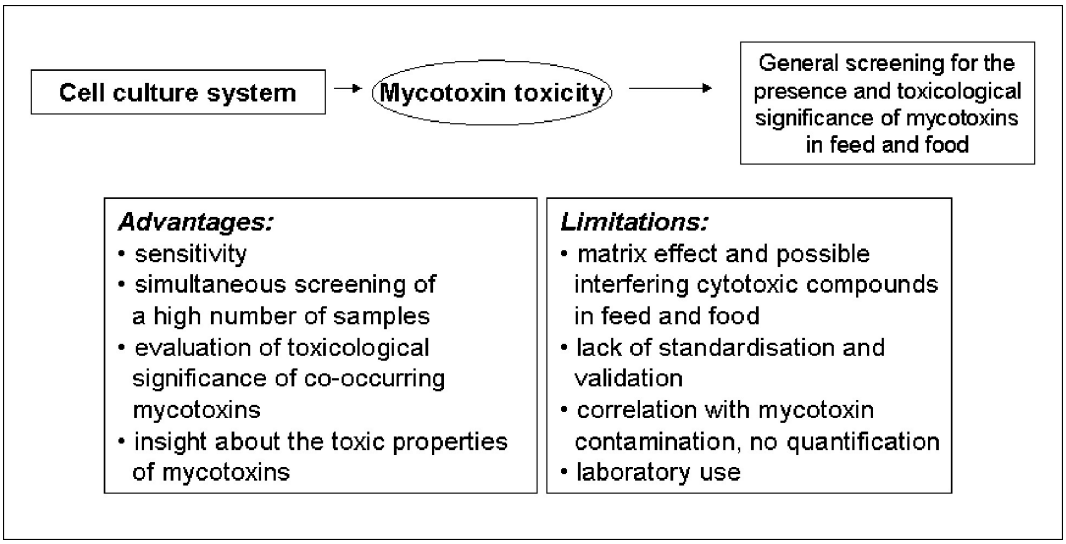

Fig. 2 - Bioassays for mycotoxin surveillance.

MITTERBAUER *et al.*, 2003). The possibilities of using cytotoxic assays, based on vertebrate cellular *in vitro* models, as rapid methods for detecting mycotoxins in feed and food have been investigated (GUTLEB *et al.*, 2002; WIDESTRAND *et al.*, 2003; CETIN and BULLERMAN, 2005). The literature concerning bioassay based on vertebrate cell lines applied to mycotoxins produced by *Fusarium* strains has been reviewed by GUTLEB *et al.* (2002). The main critical point in applying such bioassay to screen for mycotoxins, is the identification of the sensitive cell lines for a given mycotoxin. Sensitivity to mycotoxins differs greatly between cell lines. Cytotoxicity, measured as EC_{50} (half effective concentration) differed by a factor of 770 for T-2 tested in 24 cell lines (range 0.02 to 154.6 nM), 20 for HT-2 tested in 8 cell lines (range 5.9 to 176 nM), 45 for DON tested in 10 cell lines (range 135 to 1,600 nM) and 2,000 for FB_1 tested in 9 cell lines (range 0.1 to 200 μM) (GUTLEB *et al.*, 2002). LC_{50} (half lethal concentration) differed by a factor of 50 for OTA tested in 5 different cell lines (range 0.8 to 40 μg/mL) (BALDI *et al.*, 2004). These data are consistent with the findings that each mycotoxin has a main target organ *in vivo* and confirm that a sensitive cell culture model which can highly respond to the different mycotoxins must still be found. CETIN and BULLERMAN (2005) checked five different cell lines to find the most sensitive for different *Fusarium* toxins, DON, ZEN, fumonisin B1 and MON. None of the cell lines tested showed a high response to all of the *Fusarium* mycotoxins. Therefore, more than one cell line of different origin is recommended for screening purposes. The observation of both acute and chronic effects of mycotoxins in humans and animals has to be considered when bioassays are applied for qualitative and quantitative analyses of mycotoxins. The duration of the exposure period can be determined depending on the high or low acute toxicity of the mycotoxins (CETIN and BULLERMAN, 2005).

It should be noted that most of the results reported in the literature are from experiments performed using purified mycotoxin standards and only a few studies have been performed using nat-

urally-contaminated products, in which co-occurring mycotoxins may be present. Statistically designed experiments have been developed that are based on *in vitro* cytotoxicity in order to screen mycotoxin mixtures of *Fusarium* for possible interaction (GROTEN *et al.*, 1998; TAJIMA *et al.*, 2002). In this context, one advantage and promising aspect of bioassay, as a complementary approach to chemical analysis, is that the feed and food are evaluated as *"a unit"* in which the co-occurrence of mycotoxins may be important in the outbreak of the biological effect of mycotoxins (WENEHED *et al.*, 2003). Toxicity of food and feed is influenced by several factors that affect the magnitude of toxicity in humans or animals that consume mycotoxin-contaminated products (HUSSEIN and BRASEL, 2001). These factors, including species, mechanisms/modes of action, metabolism, and defence mechanisms, make it difficult to find a consistent correlation between the presence or absence of mycotoxins and toxicity. Current knowledge limits the capability of toxicity determination to toxin detection. Preliminary results from experiments performed with cellular models and naturally-contaminated products have not always been consistent and correlated with the concentration of mycotoxin tested by chemical analysis. A good correlation was found between cytotoxicity of T-2 contaminated corn extract and the T-2 concentration analysed by GC (PORCHER *et al.*, 1987). LANGSETH *et al* (1997) found that cytotoxicity of grain extract was correlated with the amount of *F. avenaceum* but not with the concentration of DON. WIDESTRAND *et al.* (2003) tested the cytotoxicity of wheat, oat and barley and found a good correlation between toxin concentration of T-2, HT-2, DON or nivalenol (NIV) and the cytotoxic effect on the cells. These authors conclude that in comparison with the chemical methodology, the sensitivity of the bioassay to trichothecenes was less than the sensitivity of GC-ECD (electron capture detection). However, a few oat samples were toxic for cells, although the trichothecene concentrations were very low. These results may indicate the presence of unknown toxin fungal metabolites in addition to those that were chemically identified; this indicates the additional value of a bioassay.

In conclusion, although positive correlations exist between mycotoxin toxicity and the mycotoxin level of contamination, the use of bioassay as an indicator for mycotoxin contamination should still be used with caution. Limitations of the currently used cell culture systems in screening procedures include the lack of standardization which is still associated with poor reproducibility and insufficient throughput and user-friendliness. Moreover, these bioassays can only be performed in a laboratory environment. The possibility of carrying out bioassays on microtiter plates provides a great advantage and allows a large number of samples to be screened simultaneously. Further implementation of automated cell culture systems may increase the use of bioassays as objective, fast, cheap large-scale screening tools for mycotoxin surveillance in order to identify samples that should be subjected to further chemical analysis.

CONCLUSIONS

Mycotoxin contamination of food and feed could have a significant effect on public health so it deserves significant attention. The food and feed industry should take the lead in these efforts, because it will provide improved economic sustainability of the industry, enhanced food safety, enhanced international trade and improved public health. Therefore, the development of low-tech, rapid and inexpensive methods for mycotoxin monitoring and surveillance is a world health imperative. In response,

several novel approaches have been developed. The need for validated rapid methods is going to increase in the near future due to the extensive monitoring programmes in and outside of the laboratory. Rapid analytical procedures are characterized by practical criteria: the speed with which the assay can be performed, the level of technical skill required to perform the assay, the qualitative or quantitative results and the *in situ* use. Most rapid methods may be a compromise, but the relative importance of each criterion must be considered according to the method used and the purpose of the analysis. A number of new techniques are emerging for rapid mycotoxin analysis in food and feed and are at various stages of development. Further progress, such as *fitness for purpose* analyses for mycotoxins, are expected through the use of technologies for rapid detection of mycotoxigenic fungi and cell culture-based bioassay. These rapid methods may provide suitable analytical tools for predicting mycotoxin contamination and associated risks within an integrated and holistic mycotoxin detection system aimed at preventing toxins from entering the food chain and minimizing consumer exposure.

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PAPER

FATE OF AFLATOXIN M_1 DURING PRODUCTION AND STORAGE OF CRESCENZA CHEESE

RIPARTIZIONE DI AFLATOSSINA M_1 NEL CORSO DELLA PRODUZIONE E CONSERVAZIONE DEL FORMAGGIO CRESCENZA

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ABSTRACT

Crescenza cheese was prepared from milk that was naturally contaminated with aflatoxin M_1 (AFM_1). Three milk batches were processed at AFM_1 levels of <5, 128 and 257 ng kg^{-1}. AFM_1 analysis was carried out using an immunoenzymatic technique (ELISA) and HPLC. After clotting, AFM_1 was analysed on both curd and whey fractions. The Enrichment Factor of the toxin in the curd over that in the milk was on average 2.56±0.13, while about 50% of the toxin remained in the whey. Cheese

RIASSUNTO

Sono state effettuate tre lavorazioni a Crescenza utilizzando latte naturalmente contaminato a due livelli di aflatossina M_1 (AFM_1=128 e 257 ng kg^{-1}) e un controllo negativo (AFM_1<5 ng kg^{-1}). La distribuzione della tossina nel corso della caseificazione è stata verificata analizzando separatamente la cagliata e il siero di spurgo, sia mediante tecnica immunoenzimatica (ELISA) che per HPLC. Il Fattore di Arricchimento nella cagliata è risultato in media di 2,56±0,13, mentre il 50% della tossina è stata rinvenu-

- Key words: aflatoxin M_1 (AFM_1), Crescenza cheese, distribution, ELISA, HPLC, stability -

samples were analysed for AFM_1 content during the 15-day storage time. The AFM_1 concentration decreased during the first 5 days and then remained constant.

ta nel siero. I formaggi sono stati inoltre monitorati per 15 giorni per verificare la stabilità della tossina durante la conservazione: nei primi 5 giorni si è registrata una diminuzione della concentrazione, mentre in seguito il livello di AFM_1 si è mantenuto costante.

INTRODUCTION

Aflatoxin M_1 (AFM_1) is a hydroxylated metabolite of aflatoxin B_1 (AFB_1), a toxic compound produced by *Aspergillus flavus* and *A. parasiticus* that grow on foodstuffs under particular conditions. It is excreted in milk by lactating animals that have consumed feed that is contaminated with AFB_1 (ELLIS *et al.*, 1991). AFM_1 has mutagenic and carcinogenic properties, so regulations have been established in many countries to protect consumers and ensure fair practices in food trade (FAO, 2004). Two standards for regulating levels of AFM_1 contamination in milk are currently applied: the USA regulation (0.5 μg kg^{-1}; FDA, 2005) and the European Union regulation (0.050 μg kg^{-1}; COMMISSION REGULATION, 2006). With regard to dairy products, the European regulation states that when applying maximum AFM_1 levels to processed products, changes in the concentration of the contaminant due to processing should be taken into account.

As a consequence of the aflatoxin crisis that occurred in Italy on maize grain in the summer of 2003, the ITALIAN MINISTRY OF HEALTH (2004) published a Note regarding hard, long-ripening cheeses, that fixed the maximum AFM_1 value at 0.45 μg kg^{-1}. As far as other types of cheese are concerned, due to the lack of known and officially recognised Enrichment Factors (EFs) for each type of cheese, the Ministry suggested that the maximum tolerable AFM_1 level be established as if all the toxin present in the milk would be concentrated in the final product (MINISTRY OF HEALTH, 2003). In order to keep the problem under control, especially when cheese is concerned, some countries have set specific regulations. For example, Switzerland fixed a maximum limit of AFM_1 in cheese of 250 ng kg^{-1} (RECUEIL SYSTÉMATIQUE DU DROIT FÉDÉRAL, 1995), regardless of the type of cheese.

When contaminated milk is used in cheese-making, AFM_1 favours casein more than whey (BRACKETT *et al.* 1982a), therefore the AFM_1 concentration is higher in the curd than in the milk. Many different results have been published regarding AFM_1 distribution during cheese production. BRACKETT and MARTH (1982a, b, c) and BRACKETT *et al.* (1982) carried out several studies on different cheeses, such as Cheddar, Processed Spread Cheese, Brick, Parmesan and Mozzarella cheese and found AFM_1 concentrations that were 4.3-, 1.9-, 1.7-, 5.8- and 8.1-times higher, respectively, than in skim milk. KIERMEIER and BUCHNER (1977a) determined the EFs for AFM_1 during cheese-making of Camembert and Tilsit and found values of 3.3 and 3.7, respectively. More recently, GOVARIS *et al.* (2001) and ORUC *et al.* (2006, 2007) studied Telemes, White Pickled and Kashar cheese-making processes and reported AFM_1 concentrations in the curd that were, respectively, 3.9-4.4, 3.6-4.0 and 2.9-3.4-times greater. BRACKETT and MARTH (1982b) dem-

onstrated the effect of emulsifying salt and/or heating used in the manufacture of Processed Spread Cheese. KIERMEIER and BUCHNER (1977b) reported that increasing the temperature of the added rennet and washing the curd could reduce the toxin concentration. BRACKETT *et al.* (1982) reported a very low enrichment of toxin in Brick cheese curd, with AFM_1 being recovered in the washing water. These results confirm the influence of cheese-making technology on AFM_1 distribution. However, a direct comparison of the literature results is very difficult, since data are influenced by many variables including the degree of milk contamination, cheese technology, method of analysis, etc.. The use of either naturally or artificially contaminated milk in experimental work can also influence AFM_1 distribution, since AFM_1-casein binding can be affected by adding a toxin to non-contaminated milk (BLANC *et al.*, 1983). The use of naturally contaminated milk in experiments is certainly preferred, because it reproduces the actual cheese-making conditions and thus the results are more reliable.

The aim of this work was to investigate the distribution and determine the EF of AFM_1 during the manufacturing process of Crescenza, a fresh Italian cheese, and to evaluate the toxin stability during cheese storage. Crescenza cheese is a typical, widely consumed Italian cheese, with a short storage time, due to its high moisture content (GHITTI and OTTOGALLI, 1986). It is a soft, mainly rennet cheese, which undergoes slow, delayed souring.

MATERIALS AND METHODS

Preparation of milk batches

Highly naturally contaminated AFM_1 milk (about 2,000 ng kg^{-1}), produced at the experimental farm of the University of Piacenza, was diluted with uncontaminated raw bulk milk (AFM_1<5 ng kg^{-1}), collected at the experimental farm of the Dairy Science Institute in Lodi, in order to prepare two batches of milk at different contamination levels ($batch_1$, about 100 ng kg^{-1}; $batch_2$, about 200 ng kg^{-1}). One batch of uncontaminated milk was used as the control for Crescenza cheese production.

Cheese production

All of the cheese-making was carried out at the experimental pilot plant of the Dairy Science Institute in Lodi. Each batch (about 15 kg) of raw whole milk was pasteurised in batch for 2 min at 70°C and then immediately cooled to 40°C. Liquid cattle rennet (80% chymosine, 20% pepsin), strength 1:10,000, (Caglificio Clerici S.p.A., Cadorago (CO), Italy) and starter culture (EZAL™, Rhodia Food, Dangé Saint Romain, FR) were added. The coagulation and acidification processes were carried out in 40 min. The curd was then cut and the whey was drained for 20 min. The curd was then placed in perforated moulds to obtain a complete draining and turned twice every 30 min. The cheese was transferred to a brining bath at 18°Bé (degrees Baumé = 220 g L^{-1} of NaCl) for 1 hour and then portioned and stored at 4°C for 15 days. Milk, curd, whey and brine were weighed on a weighing platform connected to a weighing terminal (Mettler ID1 Multirange, Mettler Instruments, Greifensee, CH), while the rennet and starter culture were weighed on a laboratory balance (Mark Electronic Balance, BEL Engineering, Monza, Italy). Milk, curd, whey, brine and cheese were sampled after different storage times to quantify the AFM_1 contents. Each cheese production was repeated twice.

Chemical and physico-chemical analyses

Cheese samples were analysed to estimate the following parameters:

- pH: according to the FIL method (International Dairy Federation, 1989);
- protein content: according to the

Kjeldahl method (International Standard ISO/TS 17837/IDF/RM 25:2008);

- fat content: according to the International Standard ISO 3433/IDF 222 (2008);

- total solids: according to official method (International Standard ISO 5534/IDF 004:2004).

AFM_1 determination

The toxin content was quantified by using a competitive Enzyme Linked Immunosorbent Assay (ELISA) kit to detect AFM_1 in milk and milk products (AgraQuant®Aflatoxin M_1 Assay 5/100, cod. COKAQ7000, Romer Labs, Singapore), according to ISO guidelines (INTERNATIONAL STANDARD ISO 14675, 2003). The assays of both milk and cheese were performed following the procedures given in the instruction booklet (Romer Labs Methods, PI-000045-1).

The milk and derived products were also analysed by HPLC methods, after immunoaffinity column (IA) clean-up (Aflatoxin Easiextract, Rhône Diagnostics Technologies, Glasgow, UK). AFM_1 in liquid products was extracted according to the method described by PIETRI *et al.* (2003): 50 g of de-fatted milk, or other liquid, were injected into the IA column and the AFM_1 was slowly eluted with 2 mL of methanol into a glass vial. AFM_1 in cheese was extracted according to the method of PIETRI *et al.* (2004): 5 g of grated cheese were dissolved in 50 mL of a 0.2% pepsin (Sigma, P-7000) solution in 0.1 N HCl and incubated at 42°C for 16 h, under magnetic stirring. The solution was then centrifuged at 4,500 x g for 15 min at 4°C, filtered on filter paper, neutralised with 1 N NaOH and purified using IA columns.

The methanol extracts from IA columns were evaporated under a stream of nitrogen, re-dissolved in acetonitrile:water (25:75, 1 mL) and filtered (Millipore Corp., Bedford, MA, USA, HV 0.45 μm) before HPLC analysis.

The HPLC system consisted of a Perkin Elmer 200 (Perkin Elmer, Norwalk, CT, USA), equipped with an ISS 200 sampling system and a Jasco FP-920 (Jasco Corp., Tokyo, JP) fluorescence detector (λ_{exc}365 nm λ_{em} 440 nm). The system was run by Turbochrom PC software (Perkin Elmer). A Supersphere 100 RP-18 column (Merck, Darmstadt, DE, 4 μm particle size, 125x4 mm I.D.) was used at ambient temperature, with a mobile phase of water:acetonitrile (75:25) at 1.0 mL/min. Amounts of standard AFM_1 (Sigma Aldrich, St. Louis, MO, USA) between 1 and 70 pg were injected and then quantified on the basis of peak areas using the Turbochrom PC software.

Data were corrected for recovery values (92.0% for milk and 96.1% for cheese). The limits of detection of the HPLC methods were 5 ng kg^{-1} for milk and brine and 20 ng kg^{-1} for cheese.

Data processing

AFM_1 concentration values were processed to obtain the following data:

- amount of toxin (ng): AFM_1 concentration (ng kg^{-1}) * amount of material (kg);

- AFM_1 distribution (%): (ng $AFM_{1\ product}$/ng $AFM_{1\ raw\ material}$)* 100;

- Enrichment Factor (EF): (ng kg^{-1} $AFM_{1\ product}$/ ng kg^{-1} $AFM_{1\ raw\ material}$)* 100.

RESULTS AND DISCUSSION

The weight data (Table 1) were used to calculate yields, mass balance and AFM_1 distribution. About 2.5 kg of cheese were obtained, with an average yield of 17%, which is on the same order of magnitude as that reported for industrial production.

Physico-chemical data related to the three different cheese-making processes (control, $batch_1$ and $batch_2$) are shown in Table 2. These results are in good agreement with the analytical data reported by GHITTI and OTTOGALLI (1986).

The AFM_1 levels determined by ELISA

Table 1 - Quantities of milk used in each trial, of rennet and starter culture added during cheese making, of curd and whey obtained after coagulation and of brine used to salt Crescenza cheese. Yield (%) is calculated as: curd (kg) / milk (kg)*100.

	Control	Batch$_1$	Batch$_2$
Milk (kg)	13.782	15.540	15.522
Starter culture (g)	0.41	0.47	0.46
Rennet (g)	10.10	10.20	11.20
Curd (kg)	2.402	2.738	2.564
Whey (kg)	10.941	13.158	13.226
Brine (kg)	13.000	20.000	22.200
Yield (%)	17.43	17.62	16.52

and HPLC in samples from the Crescenza cheese production are plotted in Fig. 1. The relationship between the two methods was evaluated; the linear regression showed a slope value of 1.017, indicating very similar results with the two techniques, but the coefficient of determination (R^2=0.903) was strongly influenced by highly contaminated samples (AFM_1 concentration > 500 ng kg^{-1}), which were inaccurately determined by ELISA. In fact, these values are far from the declared linearity range of the assay. In general, very fitting results were obtained for liquid samples, while the AFM_1 contents of cheese determined by ELISA were less correlated to the HPLC data. HPLC is generally known to give more accurate results, due to the additional clean-up step (with an immuno-affinity column) required to reduce interferences before chromatographic analysis. ELISA, which is less accurate and less precise than HPLC, is a very effective semi-quantitative screening method when a large number of samples need to be analysed.

The HPLC data were used to study the AFM_1 distribution in Crescenza cheese production. The data, reported in Table 3, show a higher concentration of

Table 2 - pH, protein, fat and total solids in Crescenza cheese made from the three different batches of milk. Data are expressed as mean ± standard deviation.

	pH	Protein (%)	Fat (%)	Total solids (%)
Control	5.20±0.02	15.36±0.51	21.09±1.14	41.49±0.84
Batch$_1$	5.15±0.02	15.67±0.16	18.58±0.89	45.78±0.40
Batch$_2$	5.03±0.02	16.58±0.30	20.06±0.66	42.24±0.61

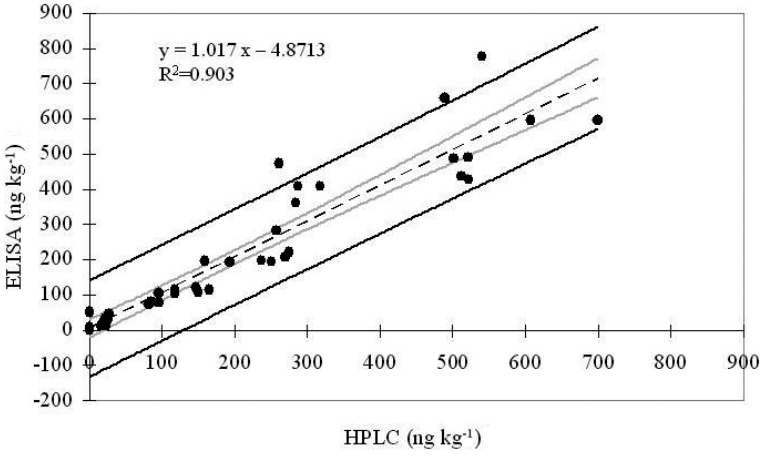

Fig. 1 - Plot of AFM_1 content, determined by ELISA and HPLC, in samples from the Crescenza cheese production (milk, curd, whey and brine samples). R^2= coefficient of determination. Dashed line: linear regression line; solid black line: 95% prediction interval of the regression line; solid grey line: 95% confidence interval of the mean.

Table 3 - HPLC data of milk, curd, whey and brine samples obtained from Crescenza cheese production (mean ± standard deviation). The concentration of AFM_1 is reported for all samples; the percentage of AFM_1 is calculated for curd and whey and their sum is indicated as AFM_1 recovery; the Enrichment Factor (EF) is only calculated for curd.

	Milk (ng kg^{-1})	Curd			Whey		Brine (ng kg^{-1})	AFM_1 Recovery (%)
		Concentration (ng kg^{-1})	EF	% AFM_1	Concentration (ng kg^{-1})	% AFM_1		
Control	<5	nd*	nc**	nc	nd	nc	nd	nc
$Batch_1$	128±14	314±22	2.45	43.2	83±2	53.6	nd	96.8
$Batch_2$	257±26	680±68	2.65	43.6	149±2	47.9	nd	91.5

* nd = not detectable;
** nc = not calculable.

AFM_1 in the curd than in the milk, confirming AFM_1 affinity for casein; the AFM_1 concentrations in the whey were lower than those found in either the curd or the milk. About 43% of the toxin detected in the milk was retained in the curd, while 50% was found in the whey, regardless of the initial level of milk contamination. The Enrichment Factor (EF) for Crescenza cheese, calculated as the ratio of the toxin concentration in the product over that in the milk, was 2.56±0.13. This value, although on the same order of magnitude, differs from values reported in the literature for other types of cheese (BRACKETT and MARTH, 1982b, c; BRACKETT *et al.*, 1982; KIERMEIER and BUCHNER 1977a, b; GOVARIS *et al.*, 2001; ORUC *et al.*, 2006, 2007). In Crescenza cheese production, the effect of brine on AFM_1 concentration in curd seems to be in contrast with the results reported by others (KIERMEIER *et al.*, 1977b; BRACKETT *et al.*, 1982; GOVARIS *et al.*, 2001; ORUC *et al.*, 2006, 2007). In fact, no trace of the toxin was detected in the brine, even when the cheese was produced from highly contaminated milk ($batch_2$). The amount of AFM_1 removed from the curd was very low. Therefore, it was diluted in the brining solution to a level that was lower than the detection limit of the method used. This amount could partially explain the 1.9-6.9% of

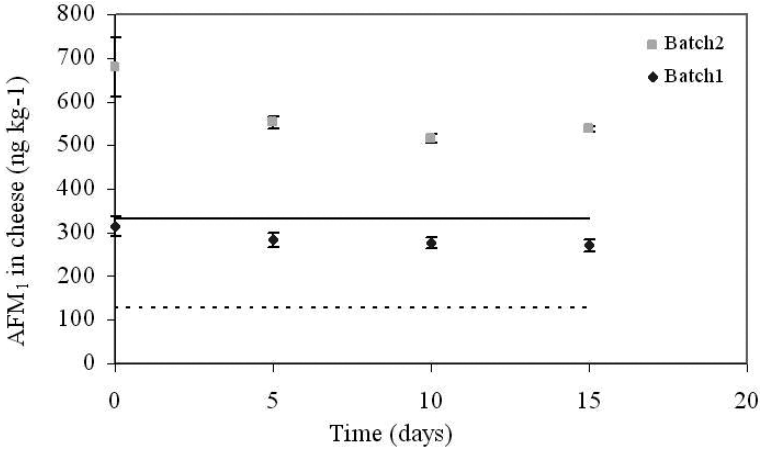

Fig. 2 - AFM_1 level reported in legend in Crescenza cheese during storage. Samples were collected at 5 day intervals from 0 to 15 days. Solid line indicates the maximum AFM_1 level (333 ppt) according to the Note of the Italian Ministry of Health (2003, N. 609/1774/388); dashed line indicates the maximum AFM_1 level as calculated according to the experimental Enrichment Factor (127 ppt) (see text for further explanation).

toxin that was lacking in the mass balance of the two processes.

In order to evaluate the influence of storage on toxin stability, cheese samples were analysed for AFM_1 content every 5 days for 15 days. Data are plotted in Fig. 2. The level of AFM_1 in cheese decreased during the first 5 days, and then it remained constant. These results are in agreement with other authors (BRACKETT and MARTH, 1982a, b, c; BRACKETT *et al.*, 1982; KIERMEIER and BUCHNER 1977a, b; ORUC *et al.*, 2006, 2007) who reported an overall stability of AFM_1 during ripening and storage. The volume of the whey drained during storage was small and its AFM_1 content ranged from 5 to 50 ng kg^{-1}(data not shown); this draining did not explain the decrease in the AFM_1 level observed in the first 5 days. This decrease could be the result of a partial degradation or an induced change in casein-AFM_1 binding, but it is not clear which factor(s) influenced the toxin recovery.

The EFs data are very important to gain information on AFM_1 distribution during manufacturing and to establish limits in cheese. In the Note published in 2004, the Italian Ministry suggested using coefficients of processing from milk to cheese, which are reported in Appendix 2 of the decree of the ITALIAN MINISTRY OF AGRICULTURE (2003), using the following formula:

Maximum accepted level of AFM_1 in cheese = maximum accepted level of toxin in milk · Coefficient of Transformation.

According to this formula, the maximum accepted level for Crescenza cheese would be about 0.30 μg kg^{-1} (0.050 * 6.66 = 0.33 μg kg^{-1}). If the EF characteristic for each cheese technology is considered, the formula would be:

Maximum accepted level of AFM_1 in cheese = maximum accepted level in milk · Enrichment Factor.

In the case of Crescenza cheese, the proposed maximum level would be about 0.13 μg kg^{-1} (0.050 * 2.55 = 0.127 μg kg^{-1}), which is a far more restrictive limit. In Fig. 2 the two limits, calculated according the Note of the Ministry and using the experimental EF, are reported. It is evident that Crescenza cheese produced with milk containing about 100 ng kg^{-1} of AFM_1 should be accepted according to the ministerial decree, while it should be rejected based on the experimental EF according to the EC Regulation.

CONCLUSIONS

The EF value for Crescenza cheese, although on the same order of magnitude, differs from those reported in the literature for other types of cheese. This confirms the influence that cheese-making technology has on AFM_1 distribution. Further investigations should be conducted to study the nature of the binding of AFM_1 to casein and the effect that several technological factors have on the binding. The factor(s) that influence the toxin recovery also need to be explained.

Concerning the maximum levels allowed for dairy products, this study stresses the importance of establishing specific EFs for each type of production, in order to set more restrictive and more realistic limits in comparison with those currently suggested by the law. This would protect consumers against the risk of ingesting highly contaminated products. It is also necessary to standardize the limits of the toxin content in food in order to ensure fair food trade practices.

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PAPER

PREVALENCE OF *MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS* INFECTION IN YOUNG BEEF ANIMALS SUBMITTED TO A REGIONAL ABATTOIR

PREVALENZA DELL'INFEZIONE DA *MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS* IN ANIMALI DA CARNE MACELLATI IN UN MATTATOIO REGIONALE

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ABSTRACT

Different diagnostic methods (ELISA, faecal culture, tissue culture and gross pathology) were used to detect beef cattle infected with *Mycobacterium avium* subsp. *paratuberculosis* at the slaughter house. Since this bacterium has been linked to human Crohn's disease, it is important to assess the potential risks posed by beef consumption. One-hundred four head of cattle between the ages of 12 and 24 months were tested in an EU slaughter house. The overall prevalence of animals in-

RIASSUNTO

Diversi metodi diagnostici (coltura fecale, coltura dei tessuti, test sierologico ed esame macroscopico dell'intestino) sono stati impiegati al fine di individuare al mattatoio bovini da carne infetti da *Mycobacterium avium* subsp. *paratuberculosis*. Questo microrganismo è sospettato essere legato al morbo di Crohn dell'uomo ed è perciò importante determinare il rischio di infezione rappresentato dal consumo di carne bovina. È stata studiata la prevalenza dell'infezione in 104 bovini da carne di

- Key words: beef cattle, *Mycobacterium avium* subsp. *paratuberculosis*, slaughterhouse -

fected with *Mycobacterium avium* subsp. *paratuberculosis* found by combining the results obtained by culturing the faeces, followed by confirmation of the positive samples by using PCR and serological tests was 11.54%. The isolation of *Mycobacterium avium* subsp. *paratuberculosis* from organ cultures in animals that tested negative in the standard testing methods suggests that the incidence of infection may be underestimated.

età compresa tra 12 e 24 mesi, macellati in un mattatoio CE. La prevalenza complessiva della paratubercolosi, ottenuta combinando i risultati della coltura fecale seguita da conferma tramite PCR e dell'esame sierologico, è pari all'11,54%. Inoltre, l'isolamento di *Mycobacterium avium* subsp. *paratuberculosis*, mediante coltura, da organi di animali risultati negativi ad entrambi i test impiegati lascia pensare che la reale incidenza dell'infezione possa essere sottostimata tramite l'utilizzo dei metodi standard di identificazione.

INTRODUCTION

Paratuberculosis or Johne's disease, caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP), is an intestinal disease that primarily infects the ileum and related lymphoid tissues of wild and domestic ruminants (HARRIS and BARLETTA, 2001; LINNABARY *et al.,* 2001; AYELE *et al.,* 2004). Although closely related to *M. bovis* and *M. tuberculosis*, that cause tuberculosis in cattle and humans, respectively, MAP causes a different kind of disease that is characterised by a very long incubation period. Although it is generally accepted that infection occurs at a very early age through contact with infected faeces or milk (LINNABARY *et al.,* 2001; TIWARI *et al.*, 2006), or even in the uterus (SWEENEY *et al.,* 1992), the clinical symptoms rarely appear before the animal is at least 2 years old (REICHEL *et al.,* 1999; LINNABARY *et al.,* 2001). Infection can take place later in life, but it is less common since resistance increases with age as substantiated by the difficulty to experimentally establish an infection in adult animals (SWEENEY, 1996). It is easier to detect the disease in dairy herds, because the cows are older and clinically evident cases can be found more easily than in beef herds. MAP infection causes economic losses on dairy farms because even subclinically-infected cows have a lower milk production and display weight loss and decreased fertility (HUTCHINSON, 1996; STABEL, 2000; TIWARI *et al.,* 2006). Cows are culled when typical clinical signs appear (HUTCHINSON, 1996; COLLINS *et al.,* 2005).

MAP is of particular interest because of the possibility that it is involved in the etiopathogenesis of Crohn's disease, a chronic granulomatous ileocolitis in humans (CHIODINI *et al.,* 1996; COLLINS *et al.,* 2000; El-ZAATARI *et al.,* 2001; BULL *et al.,* 2003). This disease is somewhat similar to paratuberculosis in cattle in terms of the type of lesions (the ileum is usually involved with granulomatous inflammation) and symptoms (weight loss, diarrhoea and abdominal pain). It may become so serious as to require surgical removal of the diseased portion and resectioning of the intestinal tract (COCITO *et al.,* 1994; CHIODINI *et al.,* 1996; SELBY, 2000). Crohn's disease appears to be a disorder of the immune response in which an exogenous or endogenous stimulus causes an abnormal inflammatory response of the intestine (CHIODINI

et al., 1996; CHAMBERLIN *et al.,* 2001). The finding of MAP in the intestines of some patients affected by Crohn's disease suggests that it may be related to one of these exogenous factors (CHIODINI *et al.,* 1996; CHAMBERLIN *et al.,* 2001; FELLER *et al.,* 2007). There is evidence that MAP is excreted in milk (STREETER, 1995; NAUTA *et al.,* 1998; LINNABARY *et al.,* 2001); beef products can be contaminated through the blood stream and after slaughter, through faecal contamination (ROSSITER and HENNING, 2001; COLLINS, 2003; AYELE *et al.,* 2004; GRANT, 2006; ABUBAKAR *et al.*, 2007). Therefore it is of great importance to know how prevalent MAP infection is in beef and dairy herds in order to control the potential zoonotic risk.

The very nature of the organism and the disease process present problems in the testing systems used to detect MAP (COLLINS *et al.,* 2005; MCKENNA *et al.,* 2005; SWEENEY *et al.,* 2006; TIWARI *et al.,* 2006; ROUSSEL *et al.,* 2007). The aim of this study was to use various detection methods to determine the prevalence of MAP infection in various cattle breeds that are routinely presented for slaughter. The usefulness of macroscopic examination of intestines at the slaughterhouse level to detect MAP infection was also evaluated. The presence of the bacterium in various organs, some of which are consumed by humans, was determined to evaluate the risk of exposure to this potential human pathogen.

MATERIALS AND METHODS

Faeces and blood samples from 104 head of cattle between the ages of 12 and 24 months were collected at an EU abattoir in the region of Umbria Italy to determine how many were shedders or serologically positive for MAP. The intestines particularly, the ileum of the same animals, were macroscopically examined. The animals were classified as potentially positive, doubtful or negative for paratuberculosis on the basis of the presence and extent of the lesions on the intestinal mucosa, particularly the ileum. Corrugation, thickening or a granulomatous appearance are often associated with cases of paratuberculosis enteritis (COCITO *et al.,* 1994). For this reason the thickness of the ileum was measured and a threshold value was established; if the value was <0.9 cm it was doubtful that the animals were infected. If the value was >0.9 cm the animals were considered positive. Animals with no lesions were classified as negative.

The 104 animals sampled belonged to the following breeds: 47 Chianina; 22 meat-crosses; 16 Limousin; 7 Charollaise; 4 Simmenthal; 4 Friesians; 2 Marchigiana; 1 Podolica and 1 Belgian Blue. The Chianina breed predominated because it is the most common breed in this region of Italy. At the *ante-mortem* inspection, none of the above animals showed any sign of Johne's disease; some cases of diarrhoea were observed but this is not unusual in animals at lairage.

Tissue samples were collected from 14 animals that had intestinal lesions. The samples were taken from the ileum (ileocecal valve), liver, kidneys, and spleen and from the hepatic, retropharyngeal, mediastinal and deep cervical lymph nodes. The aim of this sampling was to test for the presence of MAP in the organs and to assess how many of the infected animals had been previously identified by the combined use of ELISA and faeces culture.

After the faeces and tissue samples were taken they were immediately placed in a cooler and kept at -20°C until analysed. Blood samples were centrifuged (2,500 g for 10 s) and the sera were separated and frozen at -20°C within 36 h of sample collection.

After thawing, faeces samples were used to isolate MAP according to the

NADC protocol described by STABEL (1997). A commercial Herrold's egg yolk Agar medium with Mycobactin J (HEYM) (Becton Dickinson, Cowley, Oxford, U.K.) for selective isolation of MAP was used; it was incubated at 37°C for 7 months. HEYM slopes were visually examined weekly for the first 2 months and then every 15 days; mould contaminated test tubes were discarded. All faeces samples were plated in duplicate and a MAP positive control was used.

For each organ tissue sample, the surrounding fat and connective tissue were removed before being seared with a heated spatula. Approximately 5 g of material were then collected from the inner part of the organ tissue using sterile forceps and scissors and placed in a stomacher bag with 2 mL of sterile phosphate buffer solution (PBS). The bag was placed in a stomacher (Stomacher 400 circulator, PBI international, Milano, Italy) and homogenised for 5 min. The homogenate was then poured into sterile test tubes and the same volume of 5% sulphuric acid was added for decontamination purposes. The test tube was then placed in an incubator at 37°C for 12 min. After this, the homogenate was then diluted with PBS sterile buffer to a 50 mL volume and centrifuged at 1,700 g at 4°C for 10 min; the supernatant was discarded. Sterile PBS (3 mL) and 3 drops of bromothymol blue, a pH indicator, were added to the test tube. A yellow colour appeared indicating the acidity of the suspension. Sodium hydroxide (4%) was added dropwise until a neutral pH was reached (the solution turned light green). This solution was again centrifuged at 1,700 g at 4°C for 10 min, the supernatant was discarded and the pellet obtained was diluted with 3 mL of sterile PBS. This solution was then plated in duplicate onto same culture medium used for the faeces. The plates were read as described for the faeces samples.

Suspect MAP colonies found on solid culture media that had been plated either with faeces or organ samples were confirmed by IS900 PCR. Single colonies were picked off and suspended in 100 μL of sterile deionised water, vortexed for 1 min and the suspension was heated to 100°C for 15 min to release the DNA. Lysed DNA (5 μL) from the HEYM culture was used for PCR reaction (BRANCIARI *et al.*, 2004), with primers detecting the IS900 insertion sequence that is specific for MAP (COLLINS *et al.,* 1993).

Sera samples were analysed using a commercially available ELISA kit (IDEXX Laboratories, Inc., Westbrook, Maine, USA). Following the manufacturer's recommendation, animals with a sample to positive (S/P) ratio of 0.30 or greater were defined as positive, while samples with an S/P ratio between 0.15 and 0.30 were classified as doubtful.

RESULTS

The macroscopic evaluation of the intestines of the 104 animals examined is reported in Table 1. The results are divided into two groups, the "Chianina" and the "Other breeds" that include the meat-crosses and all the other non-Chianina breeds.

Table 2 shows the animals that were MAP-positive in either the ELISA or faeces culture tests. The results of the tests that were carried out on the 14 animals whose tissues were sampled and then subjected to culture followed by PCR

Table 1 - Macroscopic classification of paratuberculosis-like lesions in bovine intestines at an EU slaughterhouse.

Cattle Breed	Negative	Doubtful	Positive
Chianina	3 (6.4%)	5 (10.6%)	39 (83.0%)
Other breeds	7 (12.3%)	18 (31.6%)	32 (56.1%)
Overall	10 (9.6%)	23 (22.1%)	71 (68.3%)

confirmation of suspect colonies, are also reported.

Table 3 reports the overall prevalence of cases in which paratuberculosis (Elisa and faeces culture) or paratuberculosis like lesions (gross pathology) were found; the prevalence values obtained for the two groups (Chianina and Other breeds) are also given. In order to determine a possible breed effect, the preva-

Table 2 - Test results for animals that tested positive to MAP in at least one of the methods used and animals whose organs were sampled.

Breed	Age (months)	Macroscopic classification[a]	Blood ELISA	Faeces culture	Tissues culture
Belgian Blue	16	+/-	-	+	N.T.
Chianina	14	+	+	-	N.T.
Chianina	17	+/-	+/-	-	N.T.
Chianina	21	+	-	-	Spleen; intestine
Chianina	23	+	-	-	Spleen; intestine
Chianina	24	+/-	-	-	Liver; intestine
Chianina	24	+	-	-	Intestine
Chianina	24	+	-	+	N.T.
Chianina	24	+	-	+	Kidney; intestine
Charollaise	16	+	-	-	-
Charollaise	16	+	-	-	Intestine
Charollaise	17	+	-	+	-
Charollaise	22	+	-	-	-
Friesian	12	+/-	-	-	Liver; intestine
Friesian	24	+	-	-	Intestine
Limousine	17	+	-	+	-
Limousine	20	+	+	-	Intestine
Meat cross	16	+	-	+	N.T.
Meat cross	19	+/-	-	+	N.T.
Meat cross	21	+	-	+	N.T.
Meat cross	24	+	-	-	Intestine
Podolica	19	+	-	+	N.T.

[a] +: positive; -: negative; +/-: doubtful.
N.T.: Not Tested.

Table 3 - Prevalence of MAP positive animals obtained with the various tests.

GROUP/TEST	Macroscopic Classification (+ and +/-) (CI 95%)	Faeces culture (CI 95%)	Blood ELISA (positive and doubtful) (CI 95%)	Faeces culture + blood ELISA (CI 95%)
Chianina	93.62% (81.44-98.34)	5.00% (0.87-18.21)	4.26% (0.74-15.73)	8.51% (2.76-21.27)
Other breeds	87.72% (75.71-94.51)	13.73% (6.15-26.87)	1.75% (0.09-10.63)	14.04% (6.68-26.35)
Overall	90.38% (82.62-95.04)	9.89% (4.9-18.4)	2.88% (0.75-8.81)	11.54% (6.73-19.66)

lence values obtained for the two groups using gross pathology and faeces culture (separately or combined) were compared using the χ^2 test; there were no significant differences. No between-group comparison was made for the ELISA results due to the low number of positive results that were found.

The results of the three paratuberculosis evaluation methods used, macroscopic examination, serological testing and faeces culture, were compared using two-way tables; agreement was extremely low, with kappa values between 0.006 and 0.052.

In the tissues sampled in the 14 animal sub-population, the overall prevalence of paratuberculosis was 28.57% (95% CI: 9.58 to 58.00%) when the serological test and the faeces culture results were considered. The value increased to 85.71% (95% CI: 56.15 to 97.49%) if the positives values from the tissue cultures were also considered.

DISCUSSION

The results of this study show that MAP is present in the Italian beef population although it is difficult to detect the disease in young animals if the standard methods (ELISA and faeces culture) are used. This is evident in the number of tissue-positive subjects that were negative to the two standard methods. The presence of MAP in young beef animals is of interest because to date, most studies have focused on older animals as being the most likely sources of MAP transmitted from animals to humans through meat (ROSSITER and HENNING, 2001). Infected animals were found among all of the most frequently slaughtered breeds in the region of Umbria; there were no significant differences between the autochthonous Chianina breed and the other breeds. Visual examination of the intestine is a very inaccurate way to detect MAP-infected animals; it is not a reliable screening method at the slaughterhouse level.

The prevalence of animals that tested positive in the faecal culture was low (9.89%) compared to that (41.10%) reported by PAVLIK *et al.* (2000) in a study on beef cattle in the Czech Republic. While they sampled animals belonging to known paratuberculosis positive cattle herds, the sampling in this study included all the young animals slaughtered at a local abattoir during a certain period. Of the 104 faecal samples, 13 could not be taken into consideration because mould developed on the two solid culture mediums that had been plated. Mould development is a known drawback of this methodology and plating each sample in duplicate is not always sufficient, as confirmed by STABEL (1997).

The results of the serologic test showed a lower prevalence (2.88%) than the results from the faeces culture. This can be explained considering the kind of immune response induced by MAP. During the early, subclinical stages of the disease, the bacterium elicits a cell-mediated response from the host; in the later stages of infection a strong humoral response becomes dominant (STABEL, 2000). All the animals considered in the present study were young and had no clinical signs of paratuberculosis infection, therefore their humoral antibody response was probably low. This explains the small number of animals that were positive to the serologic test performed. The diagnostic sensitivity of all the tests is a function of the stage of infection. For example, Elisa sensitivity is 85% when applied to animals with clinical signs, while it is only 45%, or less sensitive, in infected but clinically normal animals (SWEENEY *et al.,* 1995, COLLINS *et al.,* 2005).

The tests used were poorly correlated; this means that animals that were positive to faecal culture were not serologically positive and vice versa. When screen-

ing for MAP infection, the tests should be used in combination instead of singularly. This would increase the probability of identifying infected and potentially subclinically infected animals, especially in young cattle.

The small subpopulation of animals that were subjected to tissue culture and the bias given by only sampling animals with intestinal lesions, limits the conclusions that can be drawn. The differences between the values of MAP occurrence obtained from the combined results of faeces culture and serological testing (28.57%) and those from tissue culture (85.71%) suggests that the real extent of MAP infection in the Italian beef population is greatly underestimated.

CONCLUSIONS

The results of this study clearly show that MAP is present in the Italian beef cattle population, and has a similar distribution in the breeds that were considered. The combined use of serologic testing and faecal culture does not identify all infected animals due to irregular shedding and low humoral response that are characteristic of the early stages of the disease. The low sensitivity of the methods has also been demonstrated by the high number of animals that tested positive in organ tissue culture but which had not tested positive in the other tests used. Given the possibility of a correlation between human Crohn's disease and MAP, these findings provide further evidence of a potential public health hazard and the need to establish a plan to eradicate paratuberculosis in native herds in order to minimize public exposure to this potential human pathogen.

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PAPER

CATECHINS AND METHYLXANTHINES IN TWENTY-THREE BLACK TEA INFUSIONS BY HPLC: CORRELATIONS WITH ASTRINGENCY

DETERMINAZIONE DI CATECHINE E METILXANTINE VIA HPLC IN 23 INFUSI DI TÈ NERO: CORRELAZIONI CON L'ASTRINGENZA

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ABSTRACT

Simultaneous detection of flavanols [catechin (C), epicatechin (EC), epigallocatechin (EGC), epicatechingallat (ECG), epigallocatechingallate (EGCG)], gallic acid (GA), theogalline, methylxanthines (caffeine, theobromine, theophylline) were performed on 23 black tea infusions. A hypersil-ODS column (4.6 mm I.D. x 250 mm) with methanol: distilled water: formic acid (19.5:80.2:0.3, v:v:v) as a mobile

RIASSUNTO

È stata effettuata la determinazione simultanea dei flavanoli [(catechina (C), epicacatechina (EC), epigallocatechina (EGC), epigallocatechingallato (EGCG)I, dell'acido gallico (GA), della teogallina e delle metilxantine (caffeina, teobromina, teofihlina) in 23 differenti tipologie di infusi di tè nero. La separazione via HPLC in fase inversa è stata effettuata con una colonna hypersil-ODS (4,6 mm I.D. x 250

- Key words: astringency taste, black teas, catechins, gallic acid, methylxanthines, theogalline -

phase was used for optimizing the RP-HPLC separation (R^2=0.9997). Total flavanols varied from 1.423 to 12.051 mg 100 mg^{-1} whereas EGCG was the major catechin in all of the infusions (0.356-5.972 mg 100 mg^{-1}) ($p<0.01$). Caffeine levels were high (4.002-8.657 mg 100 mg^{-1}) followed by theobromine and theophylline ($p<0.01$). Black tea contained 0.134-1.392 mg/100 mg of GA, and 0.156-1.241 mg 100 mg^{-1} of its ester theogalline. An 8-member trained sensory panel judged black tea infusions for astringency. The correlations between the total catechins and astringency (y=1.675+6.631 ln X) (R^2=0.9551) were extremely high at the 95 percent confidence level (X=total catechin, *y*=astringency) and total interactions on astringency were obtained.

mm) e con una fase mobile costituita da metanolo:acqua distillata:acido formico (19,5:80,2:0,3; v:v:v). II contenuto in flavonoidi totali variava da 1,423 a 12,051 mg 100 mg^{-1}, mentre l'EGCG risultava essere la principale catechina in tutti gli infusi (0,356-5,972 mg 100 mg^{-1}) ($p<0,01$). I livelli di caffeina erano elevati (4,002-8,657 mg 100 mg^{-1}), seguiti dai livelli di teobromina e teofillina ($p<0,01$). Il tè nero conteneva 0,134-1,392 mg/100 mg di GA e 0,156-1,241 mg 100 mg^{-1} del suo estere teogallina. Un panel sensoriale allenato, costituito da 8 membri, giudicava le infusioni di tè nero in base all'astringenza. Le correlazioni fra le catechine totali e l'astringenza (y=1,675+6,631 In X) (R^2=0,9551) erano estremamente alte al 95% del livello di confidenza (X=catechine totali, *y*=astringenza) e sono state ottenute le interazioni totali sull'astringenza.

INTRODUCTION

Catechins or flavanols are one of the most important classes of flavonoid compounds that have strong antioxidative and anticarcinogenic effects for human health (DAVES and KEENE, 1999; LUCZAJ and SKRZYDLEWSKA, 2005). Epidemiological investigations have demonstrated that these compounds may reduce the risk of certain types of cancer and coronary heart disease (VINSON *et al.*, 1995; HIGDON and FREI, 2003). According to *in vivo* and *in vitro* studies, catechins inhibit platelet aggregation and are strong anti-inflammatory agents (KATIYAR and MUKHTAR, 1996). They have a preventive effect on the risk of chronic diseases (WOODWARD and TUNSTALL-PEDOE, 1999) and inhibit the Maillard reaction (KINAE *et al.*, 1990). Tea flavonoids have antimicrobial, antitoxin, antiviral, and antifungal activities (FRIEDMAN *et al.*, 2006; FRIEDMAN, 2007). Moreover tea catechins have an anti-ulcer effect (HAMAISHI *et al.*, 2006) and some catechins, such as epigallocatechingallate (EGCG) and epicatechin gallate (ECG) enhance insulin activity (ANDERSON and POLANSKY, 2002). Recent studies have shown that EGCG inhibited the growth of prostate cancer cells and induced apoptosis and EGCG enhanced the suppressing effect of Cd(2+) on PC-3 cells (YU *et al.*, 2007).

It is known that important levels of catechin compounds are present in green and black tea (LIN *et al.*, 1998; TOKUŞOĞLU, 2001), fruit, vegetables, some fruit juices (ARTS *et al.*, 2000ab), red and white wine (GOLDBERG *et al.*, 1998), beer (MADIGAN *et al.*, 1994), processed food (ARTS *et al.*, 2000b), cacao (ZUMBE, 1998) and chocolate (TOKUŞOĞLU and UNAL, 2002a). The

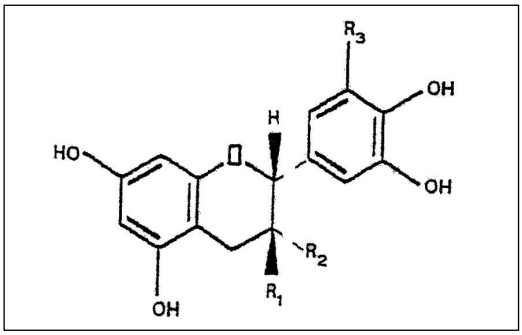


Fig. 1 - Chemical structures of flavanols (catechins).
R_1 = OH, R_2 = R_3=H, (+)-catechin (C)
R_1 = R_3 =H, R_2=OH, (-)-epicatechin (EC)
R_1 = R_3=H, R_2=galloyl, (-)-epicatechin gallate (ECG)
R_1 = H, R_2= R_3=OH, (-)-epigallocatechin (EGC)
R_1 = H, R_2=galloyl, R_3=OH, (-)-epigallocatechin gallate (EGCG)

structures of some catechin compounds are shown in Fig. 1. Tea catechins are also alternative antioxidants in meat preservation (TOKUŞOĞLU and BASMACIOĞLU, 2004).

Gallic acid is the most important phenolic acid in tea. The amount of gallic acid increases during fermentation as it is liberated from catechin gallates; it has strong antioxidant properties (LIN *et al.*, 1998) (Fig. 2a). Theogallin (5-*O*-galloylquinic acid) (Fig. 2b) is an ester of gallic acid and its presence is unique to tea, correlations of its content to the quality of black tea were suggested by LIN *et al.* (1998).

Tea contains various methylxanthines and is one of the most popular beverages in the world. In black tea, they are in the form of caffeine (1,3,7-trimethylxanthine) and the caffeine-relatives, theobromine (3,7-dimethylxanthine) and theophylline (1,3-dimethylxanthine) (TOKUŞOĞLU, 2001, 2006) (Fig. 3). The alkaloid caffeine and its catabolic products, theobromine and xanthine, exhibit both anti-oxidant and pro-oxidant properties (AZAM *et al.*, 2003).

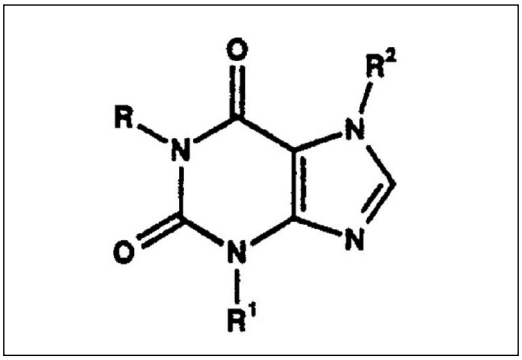
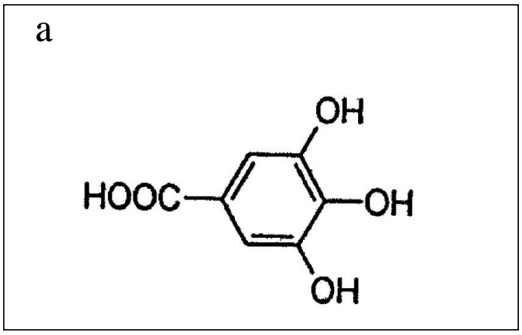


Fig. 3 - Chemical structure of alkaloids (methylxanthines)
R=R_1=R_2= CH_3, Caffeine (1,3,7-trimethylxanthine)
R=H, R_1=R_2= CH_3, Theobromine (3,7-dimethylxanthine)
R=R1= CH3, R2=H, Theophylline (1,3- dimethylxanthine)



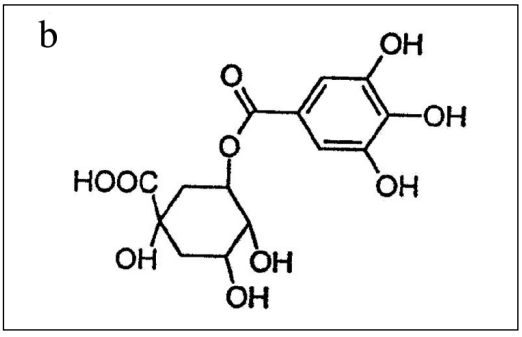


Fig. 2 - Chemical structures of (a) gallic acid and (b) theogallin.

LIN *et al.* (1998) simultaneously determined six individual catechins, gallic acid, and three alkaloids in green, pu-erh and black tea infusions using High Performance Liquid Chromatography (HPLC). Five individual flavanols, theogallin, gallic acid and caffeine, were determined simultaneously using HPLC by KUHR and ENGELHARDT (1991). It has been established that polyphenols and methylxanthines (alkaloids) account for the astringent taste (TOKUŞOĞLU, 2001, 2006; TOKUŞOĞLU and UNAL, 2002b) and so the marked influence of catechins and theaflavins on the astringency of black tea brews has been determined by HPLC (DING *et al.*, 1992).

were obtained from the Turkish Tea Board Çaykur, Lipton, Ulusoy Company. Seylan black teas were obtained from Hey Gıda A.Ş. in Turkey. The production date was the same for all of the black tea samples, all were prepared for the extraction process at the same time.

Nos.17-23 containing Çaykur No.1; Çaykur No.2, Çaykur No.3, Çaykur No.4, Çaykur No.5, Çaykur No.6 and Çaykur No.7 were unblended black teas from the Çaykur Tea Board of Turkey. Filiz, Kamelya, Rize, Rize-Turist and Burcu belong to the Çaykur Group Teas and are made up of the above-mentioned unblended teas in the percentages shown below (TOKUŞOĞLU, 2001).

'ÇAYKUR' BLACK TEA	TEA PACKAGE (g)	Çaykur No.3 %	Çaykur No.1 %	Çaykur No.2 %	Çaykur No.4 %	Çaykur No.5 %	Çaykur No.6 %	Çaykur No.7 %
Filiz	500 g	5	45	10		40		
Kamelya	500 g					75	15	10
Rize	500 g	5	40	10		45		
Rize Turist	500 g		5	10		65	15	5
Burcu	125 g		30	30	40			

The objective of this study was to develop an effective way to simultaneously determine individual catechin compounds (C, EC, ECG, EGC, EGCG), gallic acid, theogallin (gallic acid ester) and three alkaloids (caffeine, theobromine, theophylline) in black tea infusions using HPLC with UV detection, to optimize the isocratic elution procedure for chromatographic determination and to determine the influence of these compounds on astringency.

MATERIAL AND METHODS

Tea samples

Twenty-three commercial black teas (*Camellia sinensis* L.), manufactured pure black tea samples and tea bags

Standards

(+)-Catechin (C), (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epigallocatechingallat (EGCG) standard and gallic acid (GA), theobromine (3,7-dimethylxanthine) (TB), theophylline (1,3-dimethylxanthine) (TP), caffeine (1,3,7-trimethylxanthine) (CAF) were purchased from Sigma Chemical Co. (St Louis, MO, USA). (-)-Epicatechin gallate (ECG) standard was obtained from Carl Roth GmbH & Co. KG, (Karlsruhe, Germany). Methanol (HPLC grade) and formic acid were purchased from E. Merck Co. (Darmstadt, Germany).

Calibration

All standards were dissolved to 10 mg/3 mL with methanol (HPLC grade)

except for EGC, ECG, CAF, TB and GA. EGC was dissolved to 5 mg/3 mL and EC was dissolved in 10 mg/0.5 mL methanol. CAF was dissolved to 10 mg/3 mL of chloroform, TB was dissolved in the same volume with distilled water. GA was dissolved to 10 mg/3 mL with acetone. After preparation, the stock solutions were stored at 4°C. For calibration, all standards were prepared as 25 μL/mL of methanol from the above-mentioned stock solutions. Calibration curves of these standards were performed ranging from 0.5 to 25 μg/mL with mobile phase. Standard compounds had linear calibration curves through the origin (R^2=0.9997).

Extraction

Black tea samples were extracted following the modified procedure of LIN *et al.* (1998). Prepared tea infusions (TSE 3907, 1993) were equilibrated at room temperature and filtered. An aliquot of filtrate was diluted with water (x 60 dilution) to give a 0.16% tea water extract (TWE). All black tea extracts were filtered through a 0.5 μm and 0.45 μm filters (Acrodisc® syringe filters, Sigma-Aldrich, Germany), prior to injection into the HPLC.

HPLC conditions

Black tea samples were analyzed by Reversed Phase-High Performance Liquid Chromatography (RP-HPLC) using UV detection. For the analytical isocratic method, the RP-HPLC conditions were: 5 μm Hypersil-ODS column (250x4.6 mm) [Phenomenex, Torrance, CA, USA] used with the mobile phase mixture of methanol/bi-distilled water/formic acid (19.5/80.2/0.3 v/v/v). Flow rate was 1.0 mL min^{-1}. HPLC equipment was supplied by Hewlett-Packard (GMI Inc., Ramsey, MN, USA) and consisted of the following units: HP 1050 ChemStation Software, HP Interface Unit model 35900, HP 9000 Series 300 computer, HP DeskJet 500 Printer. A Waters 486 Tunable Absorbance detector (Lab X, Wilmington, NC, USA) was set at 280 nm, detector sensitivity was 0.05 A.U.F.S and the column oven was set at 40°C. A 10 μL volume was injected and total elution time was 35 min. All detections were performed from 3 separate extractions of each sample and each was injected in triplicate (n=9).

Analytical quality control

The retention times of the peaks were compared to the retention times of pure standards. Peak identity was confirmed when peak retention times were identical to those of the pure standards in mobile phase. Precision of the method was obtained within the 95% confidence limits. Recoveries were determined in duplicate in a black tea sample (Çaykur Rize) by spiking the extraction solutions (100% of the measured content) with pure standards (in range 5-25 μL) prior to sample analysis. Mean recoveries were 98.6, 100, 95.7, 97.9, 100, 99, 100, 99.8% for C, EC, EGCG, EGC, gallic acid, theobromine, theophylline and caffeine, respectively.

Sensory analysis

The sensory assessments for tea astringency evaluations were conducted by an 8 member trained sensory panel consisting of graduate students, academic (professors, researchers) and other university personnel, 24-40 years of age, from the Food Engineering Dept. of Ege University. Astringency was scaled from 1 to 9 by descriptive sensory analysis according to DING *et al.* (1992) and PIGGOTT *et al.* (1998). The panelists were trained about the astringency attributes of teas. In each session, tea samples were given as extremely astringent and less astringent references. The panelists were asked to assess each tea sample in relationship to the reference sample.

The sensory analysis was undertaken in a special sensory room that was kept dark and only a yellow light was given to each taster. By using dark pink cups (40 mL), the effect of tea brew colour was excluded. A scale of 1 to 9 was used to score astringency: 1=absent; 3=slightly astringent; 5=moderately astringent; 7=very astringent; 9=extremely astringent.

Black tea samples were ground according to TS-1561 (1990) and were infused using tea equipment called "çay yedeği" based on Turkish Standard Institute (TSE 3907, 1983). One-hundred mL of boiled water was added to 2 g of tea and brewed for 6 min. The infusions were cooled to 40°-45°C and used for tasting. Special definitions according to TOKUŞOĞLU (2001) were used for less astringency and more astringency.

Each group of samples was evaluated in one session each day (afternoon 1^{30}-2^{00} p.m.). All panelists participated in 8 sessions, three samples were evaluated per session and duplicate analyses were carried out in each session (n=2). The interval between the samples was 4 min. Good quality water, the same as the brewing water (pH = 7.12, brand "*Erikli*") and unsalted-unsweetened bread sticks (galeta) were used to neutralize the previous sample.

Statistical analysis

Data were analyzed with Statistica Ver. 6.0 for Windows (STATISTICA, 1998), by a one-way analysis of variance (ANOVA) and Multiple Regression Analysis (MRA) with respect to correlations between astringency and total catechin and alkaloid compounds.

RESULTS AND DISCUSSION

Fig. 4a shows a typical standard chromatogram of the simultaneous determination of catechins, gallic acid, theogallin and the methylxanthines (alkaloids), caffeine, theobromine and theophylline. Figs. 4b and 4c show good separation of the above-mentioned compounds in two black tea samples. Using this chromatographic method, on 23 black tea sam-

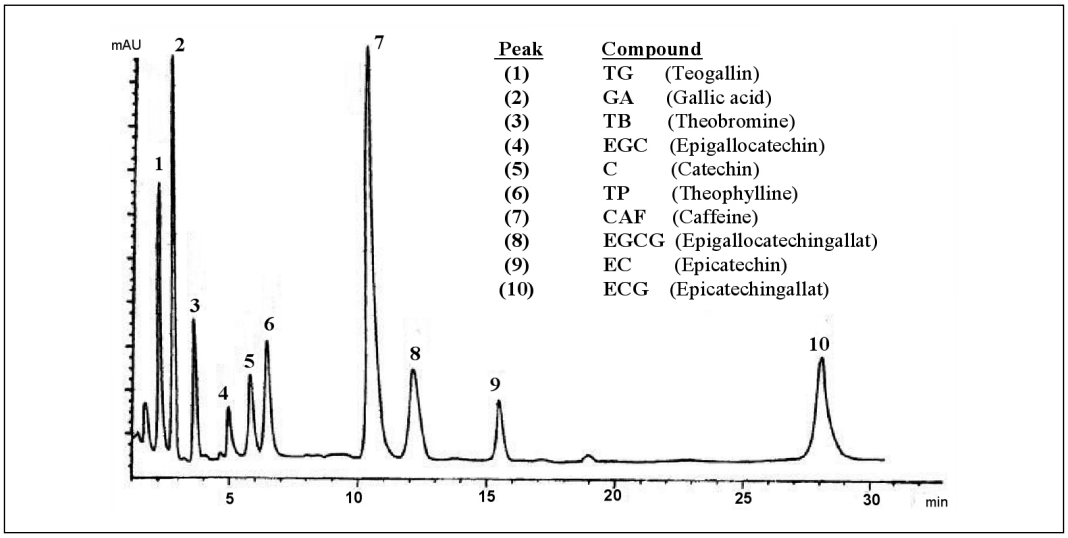


Fig. 4a - Standard chromatogram of simultaneous detection of catechins, gallic acid, theogallin and methylxanthines.

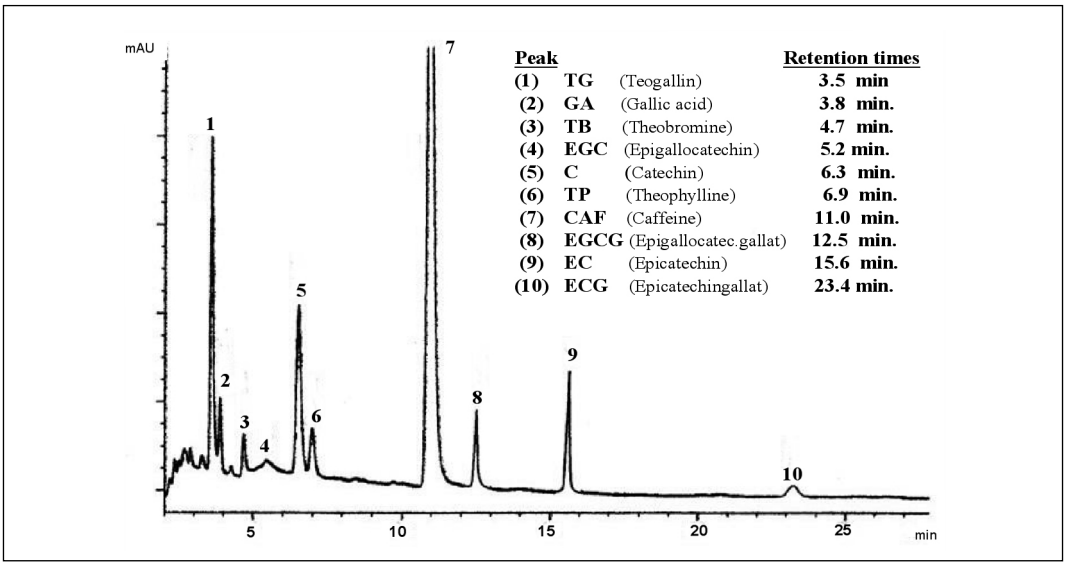


Fig. 4b - HPLC chromatogram of "*Lipton Earl Grey*".

ples (Table 1), individual flavanol (catechin) compounds [catechin (C), epicatechin (EC), epigallocatechin (EGC), epicatechingallat (ECG), epigallocatechingallate (EGCG)], gallic acid (GA), theogallin (TG) and methylxanthine (alkaloid) compounds (caffeine, theobromine, theophylline) were determined quantitatively. According to the HPLC data, in 23 black teas, 0.107-0.286 mg 100 mg^{-1} of C, 0.114-4.982 mg 100 mg^{-1} of EC, 0.260-4.297 mg 100 mg^{-1} of EGC,

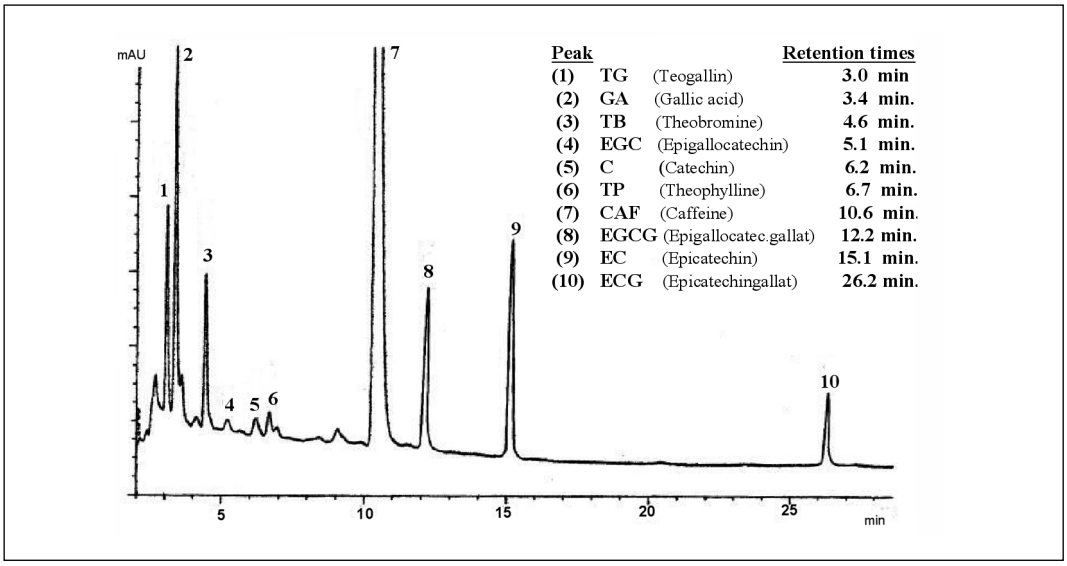


Fig. 4b - HPLC chromatogram of "*Green Island Süzme Tea Bag*".

Table 1 - Catechins, gallic acid and theogallin compounds of black teas (as mg/100 mg)*.

N.	Sample name	TG	GA	C	EC	EGC	EGCG	ECG	∑Flavanol
1	*Çaykur Filiz*	0.267± 0.01	0.244± 0.01	0.161±0.02	0.505±0.04	0.357±0.03	0.877±0.01	0.279±0.03	2.179±0.13
2	*Çaykur Kamelya*	0.328±0.03	0.292±0.01	0.134±0.04	0.607±0.01	0.486±0.01	1.674±0.05	0.247±0.02	3.148±0.15
3	*Çaykur Rize*	0.198±0.02	0.134±0.03	0.128±0.01	0.538±0.02	0.492±0.05	0.948±0.01	0.208±0.05	2.314±0.14
4	*Çaykur Rize Turist*	0.156±0.02	0.283±0.02	0.130±0.02	3.095±0.03	0.479±0.02	0.440±0.03	0.172±0.01	4.316 ±0.13
5	*Çaykur Burcu*	1.209±0.01	1.224±0.03	0.179±0.01	4.982±0.02	0.462±0.04	0.356±0.02	0.292±0.01	6.271 ±0.10
6	*Lipton Earl Grey*	1.241±0.03	0.242±0.02	0.286±0.02	3.174±0.03	1.356±0.02	3.182 ±0.02	0.244±0.03	8.242 ±0.12
7	*Lipton Yellow Label*	0.304±0.02	0.250±0.02	0.120±0.01	3.918±0.02	0.680±0.01	3.469±0.04	0.448±0.02	8.635±0.10
8	*LiptonY.Label/Poşet*	0.243±0.01	0.279±0.03	0.107±0.03	2.736±0.02	0.265±0.03	5.326±0.01	0.325±0.01	8.759±0.10
9	*Emirgan Naturel*	0.226±0.02	0.147±0.01	0.143±0.03	0.337±0.01	0.364±0.02	0.867±0.06	0.304±0.02	2.015±0.14
10	*Emirgan Aromalı*	0.213±0.03	0.932±0.04	0.112±0.01	0.139±0.04	0.373±0.01	1.748±0.03	0.343±0.04	2.715±0.13
11	*Green Isl. Brook*	0.468±0.03	0.405±0.01	0.109±0.01	0.707±0.01	4.298±0.01	4.621±0.03	0.821±0.04	10.555±0.10
12	*Green Isl. Gold*	0.981±0.02	1.247±0.03	0.148±0.02	4.022±0.02	0.260±0.02	3.661±0.04	1.338±0. 02	9.429±0.12
13	*Green Isl. Filiz*	0.217±0.01	0.229±0.02	0.134±0.01	1.377±0.03	0.953±0.01	2.653±0.02	0.645±0.01	5.762±0.08
14	*Green Isl. Rize*	0.302±0.04	0.293±0.01	0.129±0.03	2.872±0.05	0.405±0.01	3.447±0.01	0.936±0.01	7.789±0.11
15	*Green Isl. Yeşil Ada*	0.398±0.01	1.153±0.01	0.120±0.02	4.282±0.02	0.445±0.02	3.867±0.01	0.325±0.03	9.039±0.11
16	*Green Isl. Süzme Poş.*	0.437±0.02	1.392±0.01	0.127±0.01	5.023 ±0.04	0.481±0.03	5.972 ±0.03	0.448±0.01	12.051±0.12
17	*Çaykur Product No:1*	0.381±0.02	0.333±0.04	0.177±0.02	1.169±0.01	0.365±0.03	3.433±0.03	0.398±0.03	5.542 ±0.12
18	*Çaykur Product No:2*	0.311±0.02	1.193±0.02	0.127±0.04	3.022±0.01	0.399±0.01	0.487±0.02	0.448±0.02	4.483±0.10
19	*Çaykur Product No:3*	0.316±0.01	1.208±0.04	0.144±0.02	0.315±0.02	0.416±0.02	0.548±0.01	0. 686±0.04	1.423±0.11
20	*Çaykur Product No:4*	0.275±0.02	0.300±0.01	0.169±0.03	2.106±0.03	0.304±0.03	0.921 ±0.04	0.405±0.01	3.506±0.14
21	*Çaykur Product No:5*	0.287±0.04	0.275±0.02	0.158±0.04	1.090±0.03	0.392±0.02	0.621±0.02	0.403±0.01	2.664±0.12
22	*Çaykur Product No:6*	0.339±0.03	0.288±0.02	0.142±0.01	0.114±0.01	0.397±0.01	0.612±0.03	0.452±0.03	1.717±0.09
23	*Çaykur Product No:7*	0.289±0.02	0.263±0.01	0.239±0.04	0.872±0.02	0.325±0.04	0.993±0.01	0.408±0.02	1.844±0.13

*Mean±SD (*n*=9).

0.172-0.936 mg 100 mg^{-1} of ECG and 0.356-5.972 mg 100 mg^{-1} EGCG were found. The total flavanols (∑ flavanols) varied from 1.423 to 12.051 mg 100 mg^{-1} (Table 1).

EGCG was the major catechin in all of the black teas (0.356-5.972 mg 100 mg^{-1}) (p<0.01). According to the amounts determined the black tea catechins were as follows: EGCG>EC>EGC>ECG>C. According to the manufacturing process and the type of black tea, the flavanol concentrations were also classified. *Green Island Brook*, *Gold* and *Süzme Tea Bag* are pure "*Yaprak*" black teas had the highest flavanol concentration. The flavanol concentrations were as follows: in blended black tea processed by the Orthodox + 3CTC (*Lipton Earl Grey, YellowLabel, Yel.Label Tea Bag*) > in pure "*Yaprak*"+ Turkish black tea processed by the Orthodox (*Green Isl. Filiz, Rize, Yeşil Ada*) > in Turkish black tea processed by the Çaykur method (*Çaykur Burcu, Kamelya, Rize, Rize Turist, Filiz*) > in Turkish black tea processed by the Orthodox (*Ulusoy naturel, aromalı*).

DING *et al.* (1992) reported that Assam, Kenya, Ceylon and China black tea brews contain 0.07-0.46 g 100 g^{-1} of C, 1.05-3.87 g 100 g^{-1} of EGC, 0.28-2.38 g 100 g^{-1}

of ECG, 0.06-0.58 g 100 g^{-1} of EC, 0.14-5.09 g 100 g^{-1} of EGCG and 2.02-12.48 g 100 g^{-1} of total catechins (∑ flavanols). The findings of this study including Turkish, Sri Lanka and Turkish plus Sri Lanka black tea brews are in good accord with data given by DING *et al.* (1992). KUHR and ENGELHARDT (1991) reported that Assam, Kenya, SriLanka, Sikkim (India) black tea brews contain 0.06-0.48 g 100 g^{-1} of C, 0.73-3.11 g 100 g^{-1} of EGC, 0.29-1.16 g 100 g^{-1} of ECG, 0.10-0.52 g 100 g^{-1} EC, 1.04-2.87 g 100 g^{-1} EGCG and 2.30-5.90 g 100 g^{-1} of ∑ flavanols. These results are lower than our findings. LIN *et al.* (1998) reported 0.02-0.25 mg 100 mg^{-1} of EC, 0.09-0.12 mg 100 mg^{-1} of ECG, 0.08-0.26 mg 100 mg^{-1} of EGC, 0.04-0.37 mg 100 mg^{-1} of EGCG in black tea brews, whereas catechin constituents were not found in these black teas. These results are also lower than our findings. Since the brewing process was similar to our study, the differences in flavanol levels may be due to the tea type, growing conditions or black tea manufacturing process, especially the fermentation step. KUHR and ENGELHARDT (1991) reported that instant black teas contained high amounts of total flavanols (10.8-14.5 g 100 g^{-1}). These levels are in accord with our results. The EGCG level was high and was the major flavanol in the above-mentioned studies as well as in our study.

According to our data, in 23 black teas, the amount of gallic acid was 0.134-1.392 mg 100 mg^{-1} (avg. 0.548 mg 100 mg^{-1}), whereas its ester theogallin (TG) was 0.156-1.241 mg 100 mg^{-1} (avg. 0.393 mg 100 mg^{-1}). The free gallic acid (GA) level was 0.18-0.45 mg 100 mg^{-1} (DING *et al.*, 1992). This value was lower than our findings. KUHR and ENGELHARDT (1991) reported 0.29-0.62 g 100 g^{-1} of GA in black tea brews; these results are in good agreement with our study. LIN *et al.* (1998) reported 0.78-2.50 mg 100 mg^{-1} GA (avg. 1.49 mg 100 mg^{-1}) in black tea samples; these results are higher than our findings. As is known, the occurrence of theogallin (5-*O*-galloylquinic acid) is unique to black tea and the correlation of its content to the quality of black tea has been suggested (LIN *et al.*, 1998). Theogallin, the gallic acid ester, is a known indicator of tea quality (TOKUŞOĞLU, 2001). The theogallin contents were the highest in "*Lipton Earl Grey*" and "*Çaykur Burcu*". Our findings in 23 black teas are in accord with the results indicated by DING *et al.* (1992) (0.37-1.93 g 100 g^{-1} of TG).

Turkish, Sri Lanka and Turkish plus Sri Lanka black tea brews had 4.002-8.657 mg 100 mg^{-1} (avg. 6.211 mg 100 mg^{-1}) of caffeine, 0.190-2.607 mg 100 mg^{-1} of theobromine (TB) and 0.088-1.079 mg 100 mg^{-1} of theophylline (TP) (Table 2).

Total alkaloids (∑ Alkaloids) varied from 4.988 to 11.373 mg 100 mg^{-1}. Caffein was the major alkaloid (methylxanthine) in all of the black teas (4.002-8.657 mg 100 mg^{-1}) ($p<0.01$). According to the amounts determined, black tea alkaloids were: caffein > theobromin > theophylline.

In a study in which the brewing time was the same (10 min) as our study, LIN *et al.* (1998) reported 5.64-16.03 mg 100 mg^{-1} of caffeine (Caf) in China, Japan, and Taiwan black tea brews; these results are in agreement with our findings.

LIN *et al.* (1998) reported 0.26-0.89 mg 100 mg^{-1} theobromin (TB) and 0.03-0.10 mg 100 mg^{-1} of theophylline (TP) in the same samples. Compared to these results, our TB and TP data values were higher.

Based on these findings, it is estimated that, in Turkey the average daily intake of flavanols through the consumption of black teas (300 mL per day) is 7.13-60.38 mg per day and the average daily intake of the above-mentioned alkaloids through the consumption of black teas (300 mL per day) is 31.12 mg Caf/day, 3.94 mg TB/day and 0.987 mg TP/day.

Table 2 - Alkaloid (methylxanthine) content in black teas (mg/100 mg)*.

N.	Sample name	Caffein	Theobromin	Theophylline	∑Alkaloid
1	*Çaykur Filiz*	5.190±0.01	0,724±0,02	0,187±0,01	6,101±0,04
2	*Çaykur Kamelya*	4.586±0.03	0,296±0,04	0,139±0,05	5,021±0,12
3	*Çaykur Rize*	4.660±0.04	0,190±0,03	0,138±0,02	4,988±0,09
4	*Çaykur Rize Turist*	6.901±0.02	0,376±0,04	0,188±0,04	7,465±0,10
5	*Çaykur Burcu*	4.002±0.01	2,258±0,02	1,079±0,03	7,339±0,06
6	*Lipton Earl Grey*	4.978±0.02	0,399±0,04	0,195±0,01	5,572±0,07
7	*Lipton Yellow Label*	5.985±0.01	0.762±0.02	0.183±0.03	6.930±0.08
8	*Lipton Yellow LabelPoşet*	6.200±0.03	1.078±0.02	0.088±0.05	7.366±0.10
9	*Ulusoy Emirgan Naturel*	6.188±0.04	0.355±0.03	0.151±0.03	6.694±0.10
10	*Ulusoy Emirgan Aromalı*	6.389±0.03	0.441±0.01	0.185±0.02	7.015±0.06
11	*Green Island Brook*	8.657±0.01	2.607±0.03	0.109±0.04	11.373±0.08
12	*Green Island Gold*	7.361±0.05	2.391±0.02	0.091±0.02	9.843±0.09
13	*Green Island Filiz*	5.081±0.02	0.467±0.01	0.137±0.02	5.685±0.05
14	*Green Island Rize*	5.347±0.02	0.560±0.01	0.125±0.03	6.032±0.06
15	*Green Island Yeşil Ada*	6.409±0.04	1.149±0.05	0.139±0.01	7.697±0.10
16	*Green Island Süzme Poşet*	8.338±0.03	1.664±0.02	0.140±0.01	10.142±0.05
17	*Çaykur Product No:1*	7.538±0.03	0.448±0.01	0.182±0.01	8.168±0.05
18	*Çaykur Product No:2*	7.029±0.02	0.350±0.02	0.194±0.02	7.573±0.04
19	*Çaykur Product No:3*	6.463±0.04	0.342±0.02	0.187±0.01	6.992±0.07
20	*Çaykur Product No:4*	5.857±0.02	0.299±0.03	0.181±0.02	6.337±0.07
21	*Çaykur Product No:5*	6.471±0.03	0.306±0.01	0.181±0.01	6.958±0.05
22	*Çaykur Product No:6*	7.040±0.01	0.342±0.02	0.179±0.01	7.561±0.04
23	*Çaykur Product No:7*	6.190±0.03	0.282±0.01	0.146±0.03	6.618±0.07

* Mean ± SD (*n*=6).

According to the manufacturing process and the type of black tea, the alkaloid concentration was the highest in Pure "*Yaprak*" black teas (*Green Isl. Brook*, *Gold, Süzme Tea Bag*).

The quantities of tea catechins and alkaloids in commercial tea samples varies with species, season, growing conditions (soil, water, minerals, fertilizers, etc.), degree of fermentation during the manufacturing process, brewing process, brewing time, ground fraction of black tea, amount of black tea, polyphenol (PPO) activity of fresh tea leaves and the proportional oxidation potential of catechins (TOKUŞOĞLU, 2001).

The method is objective and reproducible for a simultaneous quantitative identification of flavanols (EGCG, EC, EGC, ECG, C) and alkaloids (Caf,TB,TP), gallic acid (GA) and theogallin (TG) in black tea infusions.

One of the most important criteria tea tasters use in determining quality is the astringent taste. It is established that both polyphenols (flavanols) and methylxanthines (alkaloids) account for the astringent taste of black tea (TOKUŞOĞLU, 2001; TOKUŞOĞLU and UNAL, 2002).

Table 3 shows the sensory (astringency) scores of black teas. Based on the sensory panel, the most astringent teas were as follows: Green Isl. Gold = Green-Isl.Süzme Poşet > LiptonYel label poşet > Lipton Yellow label > Green Isl. Brook > Lipton Earl grey > Çaykur Burcu. Especially the moderately astringent tea was found to be Çaykur Rize Turist. It was also found that the blends of same

Table 3 - Sensory (astringency) score of black teas.

1	*Çaykur Filiz*	3.55
2	*Çaykur Kamelya*	4.77
3	*Çaykur Rize*	3.62
4	*Çaykur Rize Turist*	6.71
5	*Çaykur Burcu*	8.30
6	*Lipton Earl grey*	8.60
7	*Lipton Yellow label*	8.72
8	*LiptonYel label poşet*	8.88
9	*Ulusoy Filiz Naturel*	5.10
10	*Ulusoy Filiz Aromalı*	4.20
11	*Green Isl. Brook*	8.65
12	*Green Isl. Gold*	9.00
13	*Green Isl. Filiz*	4.67
14	*Green Isl. Rize*	4.35
15	*Green Isl. Yeşil Ada*	8.58
16	*GreenIsl. Süzme Poşet*	9.00
17	*Çaykur Product No: 1*	4.20
18	*Çaykur Product No: 2*	6.85
19	*Çaykur Product No: 3*	3.75
20	*Çaykur Product No: 4*	4.80
21	*Çaykur Product No: 5*	3.66
22	*Çaykur Product No: 6*	3.15
23	*Çaykur Product No: 7*	3.85

1 = absent; 3 = slightly astringent; 5 = moderately astringent; 7 = very astringent; 9 = extremely astringent.

brands had similar astringency owing to the fact that they had the same origin.

It is established that polyphenols account for astringency and catechin compounds play an important part in the astringent taste of black tea. The correlations between total catechins and astringency conform closely to the logarithmic function (y = 1.675+6.631 ln X) where y=sensory score and X=$\sum$ catechins (mg/100 mg) (R^2 = 0.9551) at the 95% confident level (Fig. 5).

Total interactions between astringency and caffeine, between astringency and caffeine+theobromine, between astringency and total catechins+caffeine+theobromine, between astringency and theobromine, between astringency and gallic acid were also determined as shown in Table 4.

Astringency analysis and the concentration of phenolics depend on brewing time. Boiling the tea infusions for 10 min is good for determining the phenolics according to the standard method, but real consumers do not boil tea leaves for more than a few minutes. Therefore, the astringency rating was

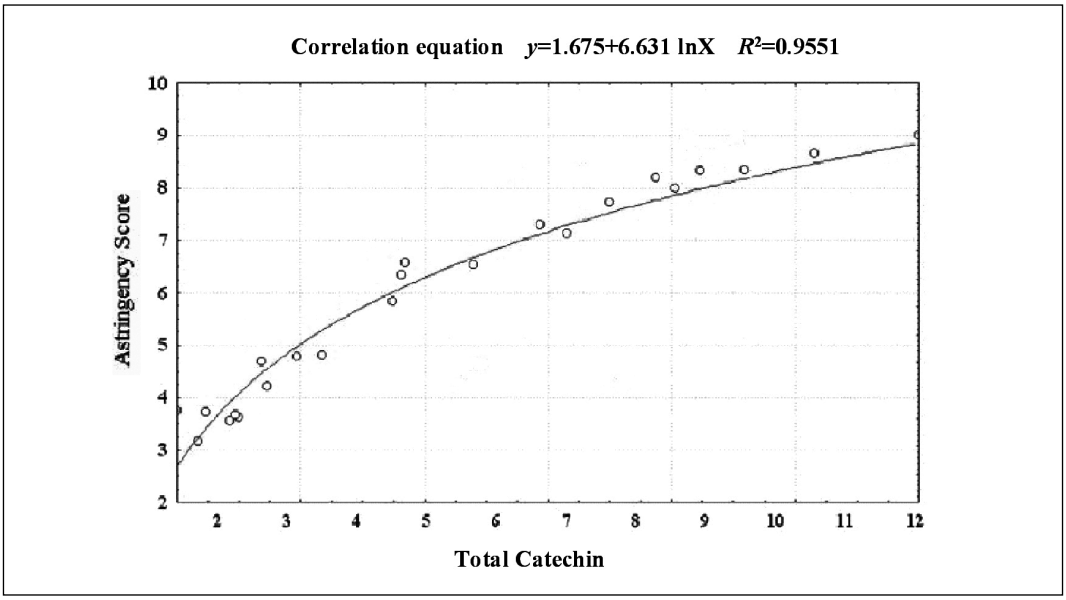


Fig. 5 - Correlation equation between total catechins and astringency [Statistica (*Version 6.0*)].

Table 4 - Correlation coefficient between alkoloids, total catechins and astringency*.

Correlations	Correlation coefficient R^2
Astr. & Caffein	0.9414[a]
Astr. & Caffein + Theob	0.9509[a]
Astr. & ∑ Catechin + Caf. + Theob	0.8891[a]
Astr. & Teobromin	0.6503[b]
Astr. & GA	0.6407[c]

[a]Highly significant; [b]Significant;
[c]Weakly significant (as $r_{0.01}$) p≤0.01.
* 95% confident level.

perhaps too high. According to DING *et al.* (1992), the correlations between total catechins and astringency also conform to a logarithmic function (y = 0.21296+2.7742 ln X) (R^2=0.91520). The correlation (R^2=0.9551) in our study was higher than that described by DING *et al.* (1992).

According to SCHARBERT and HOFMANN (2005), bioresponse-guided fractionation of black tea infusions indicate that high-molecular-weight thearubigens, the theaflavins, a series of 14 flavon-3-ol glycopyranosides and some catechins might be important contributors to black tea taste. Additional sensory studies have demonstrated for the first time that the flavanol-3-glycosides impart a velvety astringent taste sensation to the oral cavity and also contribute to the bitter taste of tea infusions by amplifying the bitterness of caffeine (SCHARBERT and HOFMANN, 2005).

HAYASHI *et al.* (2005) investigated the reductive effect of pectin on tea catechin astringency by using a taste sensor system and H-1-NMR spectroscopy. They indicated that the complexation between the gallate-type catechins and pectin is a factor that reduces catechin astringency.

The contribution of theaflavins to the astringent taste of black tea infusions was reported by SCHARBERT *et al.* (2004). The sensory analysis revealed that the theaflavins imparted a mouth-coating, astringent, and long-lasting oral sensation at the very back of the throat, and demonstrated that theaflavins have much lower oral thresholds than the astringent catechins. They also reported that when the taste activity values of the individual theaflavins were related to the overall astringency of the teas, the theaflavins accounted for less than 0.1% of the overall astringency of the teas investigated. They said that the theaflavin concentration does not seem to be a suitable measure for taste quality of tea infusions.

The result of this study in which the black tea flavanols, alkaloids and major phenolic acids were quantified and correlated with astringency are important for understanding and evaluating black tea quality and can be used in the black tea manufacturing industry. Black teas are excellent potential sources of polyphenolic flavanols and alkaloids for human consumption. The analytical method suggested is rapid, reproducible, sensitive and provided a good simultaneous separation of catechins (C,EC,ECG,EGC,EGCG), gallic acid, theogallin and methylxanthine (caffeine, theobromine, theophylline) compounds.

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SHORT COMMUNICATION

EFFECT OF ENVIRONMENTAL AND NUTRITIONAL FACTORS ON THE PRODUCTION OF BACTERIOCIN-LIKE INHIBITORY SUBSTANCES (BLIS) BY *ENTEROCOCCUS FAECIUM* STRAINS

EFFETTO DEI FATTORI AMBIENTALI E NUTRIZIONALI SULLA PRODUZIONE DI SOSTANZE BATTERIOCINA-SIMILI PRODOTTE DA CEPPI DI *ENTEROCOCCUS FAECIUM*

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ABSTRACT

Five bacteriocin-like inhibitory substances (BLIS)-producer *Enterococcus faecium* strains (WGW7.2, WGJ17.2, WGJ21.2, WGJ28.1 and WGW33.2) were characterized for their antimicrobial compound production under several growth conditions, at differ-

RIASSUNTO

Cinque ceppi di *Enterococcus faecium* (WGW7.2, WGJ17.2, WGJ21.2, WGJ28.1 e WGW33.2) in grado di produrre sostanze batteriocina-simili (BLIS) sono stati caratterizzati in diverse condizioni di crescita, con differenti concentrazioni e fonti nutriziona-

- Key words: bacteriocin-like inhibitory substances, bacteriocin production, effect of growth conditions, effect of medium composition, *Enterococcus faecium* -

ent concentrations and with different sources of nutritional factors and in the presence of ethanol and NaCl. The strains showed different behaviours. MRS and SDB were found to be the optimal media for BLIS expression. A higher BLIS production was observed at 30° and 37°C and at a pH in the neutral range. Low or no BLIS activity was detected after growth in the absence of nitrogen sources, carbohydrates, $MgSO_4$ and $MnSO_4$. Ethanol did not generally play a negative role, while NaCl determined a consistent decrease in BLIS activity that was proportional to its concentration. Due to the absence of plasmids, BLIS production by all strains was chromosomally encoded. This study is part of a more comprehensive study aimed at characterizing lactic acid bacteria (LAB) associated with the raw materials used to prepare sourdough.

li e presenza di etanolo e NaCl. I ceppi hanno mostrato un comportamento diverso; in tutti i casi, MRS e SDB sono risultati terreni ottimali per la produzione di BLIS. Alte produzioni di BLIS sono state osservate a 30° e 37°C e pH nell'intervallo di neutralità. Nessuna o scarsa attività delle BLIS è stata rilevata dopo crescita in assenza di fonti di azoto, carboidrati, $MgSO_4$ e $MnSO_4$. In generale, l'etanolo non ha mostrato influenza negativa, mentre l'NaCl ha determinato una consistente diminuzione dell'attività delle BLIS, proporzionale alla concentrazione. Non è stata rilevata la presenza di plasmidi, quindi, l'espressione delle cinque BLIS è da ritenersi codificata a livello cromosomiale. Questo studio riporta dei dati parziali relativi ad un lavoro di ricerca più ampio atto a caratterizzare i batteri lattici isolati da materie prime utilizzate per la preparazione degli impasti acidi.

INTRODUCTION

Bacteriocins are ribosomally synthesized, extracellularly released low-molecular-mass peptides or proteins (usually 30-60 amino acids) which have a bactericidal or bacteriostatic effect on other bacteria (TAGG *et al.*, 1976; KLAENHAMMER, 1988), either in the same species (narrow spectrum) or across genera (broad spectrum) (COTTER *et al.*, 2005). Bacteriocin production has been found in numerous species of bacteria, including lactic acid bacteria (LAB) which have attracted great interest in terms of food safety. LAB bacteriocins are food-grade and this offers food scientists the possibility of allowing the development of desirable flora in fermented foods or preventing the development of specific unwanted (spoilage and pathogenic) bacteria in both fermented and non-fermented foods.

Enterococci are natural inhabitants of the intestine of warm-blooded animals (DEVRIESE *et al.*, 1992) and are widely distributed on various plant surfaces (CAI, 1999). Since *Enterococcus* species generally appear and participate in food fermentation (FRANZ *et al.*, 1999), they are frequently found in different types of processed foods. Several strains of *Enterococcus faecium* produce one or more bacteriocins and some research groups have tested their potential use in food biopreservation (see FOULQUIÉ MORENO *et al.*, 2006 for a review).

Bacteriocin production is strongly dependent on growth conditions, mainly pH, incubation temperature and nutrient sources. Bacteriocin activities are not always correlated with cell concentration or the growth rate of the producer (KIM *et al.*, 1997; BOGOVIC-MATIJASIC and ROGELJ, 1998). Higher levels of

bacteriocin production often occur under conditions that differ from those required for optimal growth (PARENTE and RICCIARDI, 1994; AASEN *et al.*, 2000; TODOROV and DICKS, 2004). Supplementing the medium with growth-limiting factors, such as carbohydrates, nitrogen sources, vitamins and potassium phosphate, or adjusting the medium pH can increase bacteriocin production levels (TODOROV and DICKS, 2005).

In this study, the effects of different environmental and growth factors on the level of BLIS production by *E. faecium* WGW7.2, WGJ17.2, WGJ21.2, WGJ28.1 and WGW33.2, previously isolated from wheat (*Triticum durum*) kernels and non-conventional flours, were evaluated. This characterization is preliminary in view of the *in situ* application of the above-mentioned strains.

MATERIAL AND METHODS

Enterococcus faecium WGW7.2, WGJ17.2, WGJ21.2, WGJ28.1 and WGW33.2, isolated from sourdough raw materials (CORSETTI *et al.*, 2007) and previously characterized for their inhibitory potential against several bacteria (CORSETTI *et al.*, 2008), were cultured in MRS (Oxoid, Milan, Italy) for 24 h at 30°C. *Listeria innocua* 4202, used as indicator strain, was propagated in BHI (Oxoid) at 37°C for 24 h.

Variations in the level of BLIS production were evaluated by the well diffusion assay (SCHILLINGER and LÜCKE, 1989) with the modifications reported by CORSETTI *et al.* (2004). Experiments were carried out in triplicate with *L. innocua* 4202 inoculated at a concentration of about 10^7 CFU mL^{-1}. The antibacterial activity of the supernatants was evaluated by the critical dilution assay of BAREFOOT and KLAENHAMMER (1983). Bacteriocin activity was defined as the reciprocal of the highest dilution showing definite inhibition of the indicator strains and is expressed as activity units per mL (AU mL^{-1}).

In an attempt to find conditions for high BLIS yield, tests were performed to evaluate the effects of culture medium, temperature and pH. Cells of the producer strains were cultivated at 15°, 30°, 37° and 45°C in MRS, Sour Dough Bacteria (SDB) (KLINE and SUGIHARA, 1971), M17 (Oxoid) and a synthetic Amino Acid Assay Medium (AAAM) modified as follows: ammonium chloride and sodium chloride were omitted, dextrose and sodium acetate were reduced (5 g L^{-1} instead of 50 and 2.5 g L^{-1} instead of 40 g L^{-1}, respectively) while diammonium citrate (1 g L^{-1}), Tween 80 (0.5 g L^{-1}) and maltose (5 g L^{-1}) were added. The final pH of the medium was 6.0. Incubation was for 48 h at 30°, 37° and 45°C and for 5 days at 15°C. The effect of initial medium pH was evaluated by adjusting MRS, SDB and M17 to pH 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 with 5M NaOH or 5M HCl. Incubation was at 30°C for 48 h. Prior to inoculation [1% (v/v)] in the different test conditions, BLIS-producing strains were grown overnight at 30°C in MRS. Cultures were centrifuged at 5,000 rpm for 10 min and the resulting pellets were washed twice with sterile Ringer's solution (Sigma Aldrich Chemical Co, Milwaukee, WI, USA). The cells were then suspended, by means of a biophotometer (Eppendorf AG, Hamburg, DE), at optical density 600 nm (OD_{600}) of ca. 1.25, which roughly corresponds to a concentration of 10^9 CFU mL^{-1}.

MRS was chosen as the test medium to evaluate the effect of different growth factors on BLIS activity. The contribution of nitrogen sources was estimated by modifying MRS as follows: without nitrogen sources [yeast extract (YE), meat extract (ME) and tryptone]; with YE (without ME and tryptone); with ME (without YE and tryptone); and with tryptone (without YE and ME). MRS without sugar and with a single sugar (fructose, lactose, maltose, sucrose, ribose, mannose, xylose

and Na-gluconate) was used to evaluate carbohydrate effect. Other growth factors (triammonium citrate, K_2HPO_4, $MgSO_4$, $MnSO_4$ and Na-acetate) were eliminated from the medium composition or added at 0.5-, 2- and 5-times the typical MRS formulation from Oxoid. In order to simulate stressing food conditions, ethanol at 5, 10, 15 and 20% (v/v) or NaCl at 2, 4, 6 and 8% (w/v) were added to MRS. In the case of ethanol, it was added to the other medium ingredients and the final volume was reached with water. After each modification, the pH of the medium was adjusted to 6.0. Incubation was at 30°C for 48 h.

The Student's paired *t*-test was performed for statistical analysis of the results using a program located at http://www.physics.csbsju.edu/stats/t-test.html. The difference between BLIS production in MRS at 30°C (control) and BLIS production under the other conditions for cultivating *E. faecium* strains was considered significant at $P \leq 0.05$.

Plasmid DNA was extracted following the protocols of BIRBOIN and DOLY (1979) and ANDERSON and MCKAY (1983). A kit for plasmid extraction (Jet Quick Plasmid Miniprep Spin Kit, Genomed GmbH, Löhne, DE) was also used for rapid isolation. Plasmid curing was performed by adding acriflavine (20 μg mL^{-1}) to MRS and BLIS activity was evaluated after three consecutive propagations at 30°C for 24 h.

RESULTS AND DISCUSSION

All *E. faecium* strains grew at high levels of turbidity in MRS, SDB and M17 (OD_{600}>1), while scanty growth was observed in AAAM (OD_{600}<1). The results of BLIS activities after the development of strains in the different growth media and at different temperatures of incubation are reported in Table 1. AAAM does not contain complex nitrogen sources, hence it is a useful medium for the purification of peptides and proteins secreted by LAB. For this reason, it was chosen as a potential substitute for MRS for BLIS production by *E. faecium* strains. After growth in AAAM, no BLIS were recovered from strains WGW7.2, WGJ17.2 and WGJ21.2 and low activities were recorded for strains WGJ28.1 and WGW33.2. Thus, AAAM was not taken into account in further evaluations. SDB and M17 determined no difference in controlling the inhibition of *L. innocua* 4202 by BLIS WGW7.2 and BLIS WGW33.2, while lower activity was observed for BLIS WGJ17.2 and BLIS WGJ21.2 in both media. In the case of BLIS WGJ28.1, higher activity was due to SDB. Differences between MRS and M17 with respect to BLIS activity were statistically significant (P=0.037), while SDB did not have a significant effect on BLIS production (P>0.05). A lower production of activity in M17, compared to MRS has been reported for several bacteriocins (CHEIGH *et al.*, 2002; CHIN *et al.*, 2001; NIETO-LORANO *et al.*, 2002; TODOROV and DICKS, 2005).

Incubation temperatures different from 30°C generally had a negative effect on BLIS activity (Table 1). In the case of *E. faecium* WGJ21.2, BLIS was not recovered when grown in SDB at 45°C and almost disappeared in M17 at 45°C. Almost the same levels of BLIS were recorded from MRS at 30° and 37°C (P>0.05), suggesting that growth temperatures in that range do not play a decisive role in BLIS production. BLIS recovery was significantly (P=0.001) affected by the growth of the producer strains in MRS at 45°C. For these reasons, 30°C was chosen as the control temperature for BLIS production. It has been suggested that bacteriocin production by LAB is enhanced by suboptimal temperatures (DELGADO *et al.*, 2005; DE VUYST *et al.*, 1996). However, the results of this study are in agreement with those recorded for *E. faecium* RZS C5 for which a higher production of bacteriocin in the

range of temperature for an optimal development was observed (LEROY and DE VUYST, 2002).

The results regarding the influence of the initial pH of the culture media on BLIS activity are shown in Fig. 1. SDB exerted a positive effect in terms of BLIS production by strain WGJ28.1 and, in particular, SDB with a pH in the 6.0-8.0 range determined the maximum antimicrobial activity, even though no significant statistical difference was attributed to this medium ($P>0.05$) with MRS at pH 6.2 (control). Low pH (4.0 and 5.0) values caused a decrease in BLIS WGW7.2 in all the media, and BLIS WGJ17.2 and WGJ21.2 disappeared from SDB and M17 at pH 4.0. MRS showed no statistically significant differences ($P>0.05$) in the pH range 6.0-8.0. Regarding the influence of pH on bacteriocin expression, LEROY and DE VUYST (2002) found that the antilisterial activity of *E. faecium* RZS C5 was lower at pH values ranging from 7.5 to 8.0 than at pH values in the range 5.5-6.5. Furthermore, in the present study, an extremely high pH (9.0) significantly ($P<0.05$) (negatively) affected BLIS production in all strains grown in the three media considered.

Based on previous data (Table 1 and Fig. 1), MRS produced the best results in terms of BLIS activity in strains WGW7.2, WGJ17.2 and WGJ21.2. While SDB increased BLIS production from *E. faecium* WGJ28.1, it did not greatly influence that of strain WGW33.2. For these reasons, MRS was chosen as the model medium to study the effect of nutrients and growth factors on BLIS produc-

Table 1 - Effect of culture media and incubation temperatures on BLIS production[a].

Treatment	BLIS WGW7.2	BLIS WGJ17.2	BLIS WGJ21.2	BLIS WGJ28.1	BLIS WGW33.2
Control[b]	640[c]	640	640	2560	1280
Effect of medium[d]					
SDB	640	320	320	5120	1280
M17	640	320	320	2560	1280
AAM	-	-	-	320	320
Effect of medium and temperature					
MRS - 15°C	160	160	640	2560	640
MRS - 37°C	640	640	320	2560	1280
MRS - 45°C	320	320	160	1280	640
SDB - 15°C	160	160	160	2560	1280
SDB - 37°C	320	320	160	2560	1280
SDB - 45°C	320	160	-	1280	1280
M17 - 15°C	160	320	160	1280	640
M17 - 37°C	640	320	320	2560	1280
M17 - 45°C	320	160	80	1280	640

[a]All assays were carried out with *L. innocua* 4202 as indicator strain.
[b]BLIS activity after growth of *E. faecium* strains in MRS at 30°C for 24 h.
[c]AU mL^{-1}.
[d]evaluated at 30°C.
- no BLIS recovered.
The experiments were carried out in triplicate and the results shown are those confirmed in at least two independent measurements.

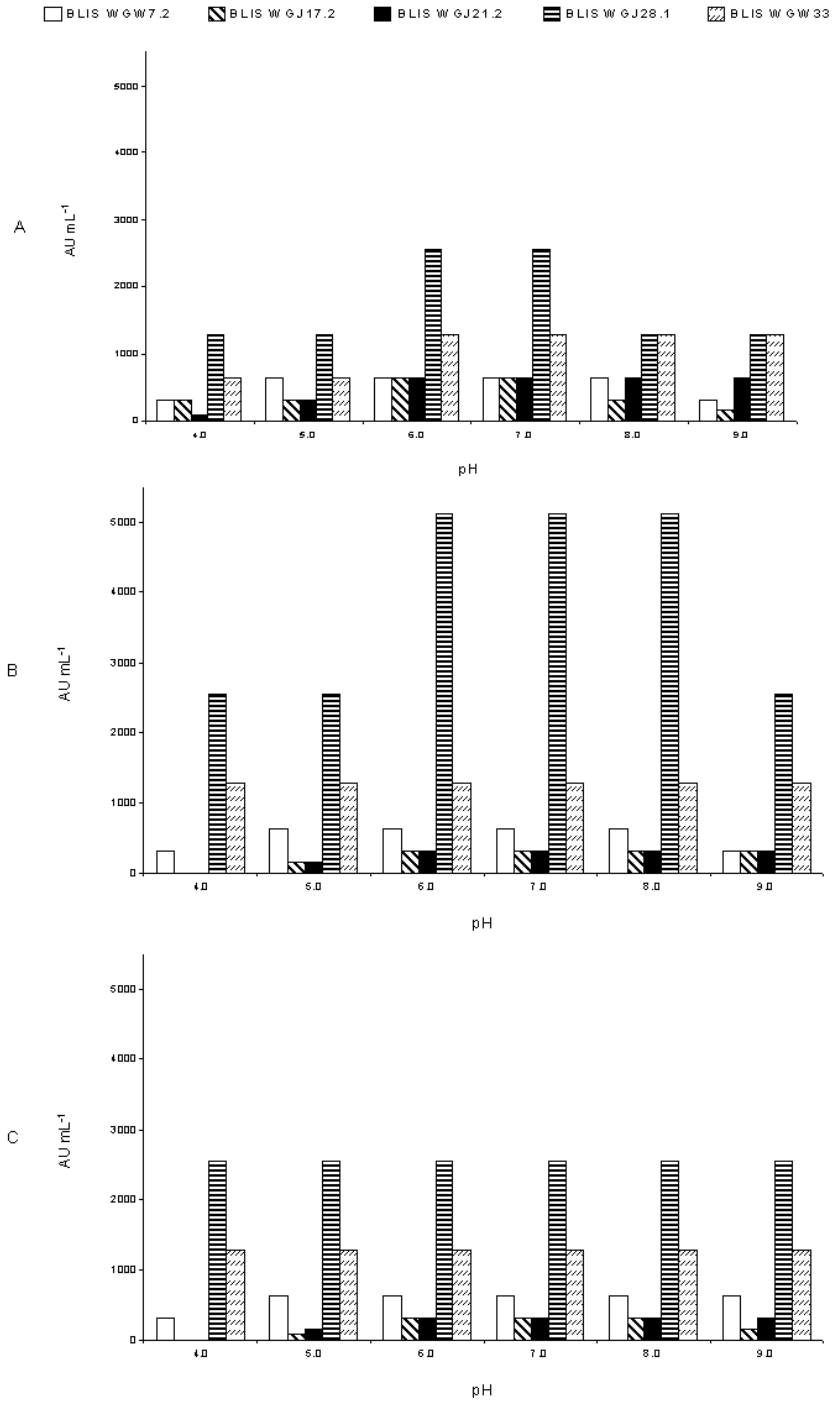

Fig. 1 - Effect of initial pH of media (A, MRS; B, SDB; C, M17) on BLIS production by *E. faecium* strains. The experiments were carried out in triplicate and the results shown are those confirmed in at least two independent measurements.

tion. In addition, MRS is often used as a fermentation medium to study bacterial growth and metabolite production by enterococci.

The results showing BLIS activity after the growth of producer strains in MRS modified with respect to nutrient composition and concentration are reported in Table 2. Statistical analysis of the data demonstrated that, except for ME (P>0.05), the lack of nitrogen sources determined consistent differences between the growth in standard MRS and modified MRS. The most negative effect was recorded in the absence of any nitrogen compound, which led to no BLIS production in strains WGW7.2, WGJ28.1 and WGW33.2 and strongly reduced BLIS activity in strains WGJ17.2 and WGJ21.2. These results are not surprising, since concentrations of nitrogen (and carbon) sources in the growth media are known to play a decisive role in the amelioration of the overall physiological response of the organism in terms of the production of antibacterial substances (PIARD and DESMAZEAUD, 1992). PARENTE and HILL (1992) found that increased nitrogen concentrations resulted in higher bacteriocin activities for enterocin 1146.

The type and concentration of carbon and nitrogen sources are the most important factors in bacteriocin production (DELGADO *et al.*, 2007). The absence of carbohydrates in MRS inhibited growth of *E. faecium* WGW7.2, WGJ17.2 and WGJ28.1. There was no BLIS produced by strain WGW33.2 and WGJ21.2 had a reduced production. Replacement of glucose with pentoses (ribose and xylose) did not influence bacterial growth, but decreased BLIS production in all the strains. Differences in BLIS recovery with lactose, maltose, Na-gluconate and mannose were not statistically significant (P>0.05), but fructose and sucrose caused a significant reduction (P=0.05 and P=0.003, respectively) in BLIS activity. These results are in contrast with those of FOULQUIÉ MORENO *et al.* (2003) who reported that the use of lactose, instead of glucose in MRS broth, increased the production of enterocin RZS C5. In addition, LEROY *et al.* (2003) reported a decrease in bacteriocin stability with low sugar levels.

The absence of Tween 80, or its presence at a concentration of 0.5 mL L^{-1} did not affect (P>0.05) BLIS production. BLIS recovery was statistically significant (P>0.05) with or without triammonium citrate, although a double concentration determined a massively higher BLIS activity with strain WGW33.2. The absence of K_2HPO_4 had a negative (P=0.037) influence on BLIS production, while its presence at higher concentrations did not show any stimulating effect. Na-acetate did not have any effect under the conditions tested. $MgSO_4$ and $MnSO_4$ did not have a significant influence (P>0.05) on BLIS production, but, when absent, the BLIS activity decreased noticeably (P=0.05). These results are in agreement with VLAEMYNCK (1996) who found that replacing $MgSO_4$ with $MgCl_2$ or K_2SO_4 in the growth medium or adding different concentrations of these salts resulted in a comparable growth and bacteriocin production by *E. faecium* RZS C5.

The effects of NaCl and organic compounds, such as ethanol, commonly found in food matrices (e.g. brined vegetables the former and bread, beer, wine, kefir, etc. the latter) were also studied. No growth (optical density values did not change from inoculation) was observed for strain WGW7.2 at 8% (w/v) NaCl, for strain WGJ17.2 at 6 and 8% (w/v) NaCl and for strain WGJ21.2 at 20% (v/v) ethanol (Figs. 2 and 3). The fact that *E. faecium* WGJ17.2 did not develop in the presence of 6% (w/v) salt is in contrast with the general characteristics of enterococci, which are known to initiate growth in broth with 6.5% (w/v) NaCl (SCHLEIFER and KIPPER-BALZ, 1984). BLIS activity decreased as the NaCl con-

Table 2 - Effect of nutrients on BLIS production[a].

Treatment	BLIS WGW7.2	BLIS WGJ17.2	BLIS WGJ21.2	BLIS WGJ28.1	BLIS WGW33.2
Control[b]	640[c]	640	640	2560	1280
N sources					
without	-	160	160	-	-
with YE (4 g L^{-1})	160	320	640	2560	1280
with ME (8 g L-1)	640	320	640	2560	1280
with tryptone (10 g L^{-1})	320	320	640	2560	1280
Carbohydrates					
without	NG	NG	80	NG	-
with fructose (20 g L^{-1})	-	-	640	2560	640
with lactose (20 g L^{-1})	640	320	640	2560	1280
with maltose (20 g L^{-1})	640	320	640	5120	1280
with sucrose (20 g L^{-1})	-	320	640	1280	640
with ribose (20 g L^{-1})	-	-	-	-	-
with Na-gluconate (20 g L^{-1})	1280	320	640	2560	640
with mannose (20 g L^{-1})	640	320	640	5120	1280
with xylose (20 g L^{-1})	-	-	-	-	-
Tween 80					
without	320	320	320	2560	1280
with (0.5 mL L^{-1})	320	320	320	2560	1280
with (2 mL L^{-1})	1280	640	1280	2560	1280
with (5 mL L^{-1})	160	640	320	1280	640
Triammonium citrate					
without	640	640	640	2560	1280
with (1 g L^{-1})	640	640	640	2560	1280
with (4 g L^{-1})	640	640	640	2560	5120
with (10 g L^{-1})	640	640	640	2560	1280
K_2HPO_4					
without	320	320	640	1280	1280
with (1 g L^{-1})	640	640	640	2560	1280
with (4 g L^{-1})	640	640	640	2560	1280
with (10 g L^{-1})	640	640	320	1280	1280
Na-acetate × $3H_2O$					
without	640	640	640	1280	640
with (2.5 g L^{-1})	640	640	640	2560	1280
with (10 g L^{-1})	640	640	640	2560	1280
with (25 g L^{-1})	640	640	1280	2560	1280
$MgSO_4 \times 7H_2O$ and $MnSO_4 \times 4H_2O$					
without both	-	-	-	2560	1280
without $MnSO_4$ with $MgSO_4$ (0.2 g L^{-1})	640	640	640	2560	1280
without $MnSO_4$ with $MgSO_4$ (0.1 g L^{-1})	640	640	640	2560	1280
without $MnSO_4$ with $MgSO_4$ (0.4 g L^{-1})	640	640	640	2560	1280
without $MnSO_4$ with $MgSO_4$ (1 g L^{-1})	640	640	640	2560	1280
without $MgSO_4$ with $MnSO_4$ (0.05 g L^{-1})	640	640	640	2560	1280
without $MgSO_4$ with $MnSO_4$ (0.025 g L^{-1})	640	640	640	2560	1280
without $MgSO_4$ with $MnSO_4$ (0.1 g L^{-1})	640	640	640	2560	1280
without $MgSO_4$ with $MnSO_4$ (0.5 g L^{-1})	640	640	640	1280	1280

[a]All assays were carried out with *L. innocua* 4202 as indicator strain. The composition of the MRS was varied.
[b]BLIS activity after growth of *E. faecium* strains in MRS at 30°C for 24 h.
[c]AU/mL.
N, nitrogen; YE, yeast extract; ME, meat extract; NG, no growth was observed; - no BLIS recovered.
The experiments were carried out in triplicate and the results shown are those confirmed in at least two independent measurements.

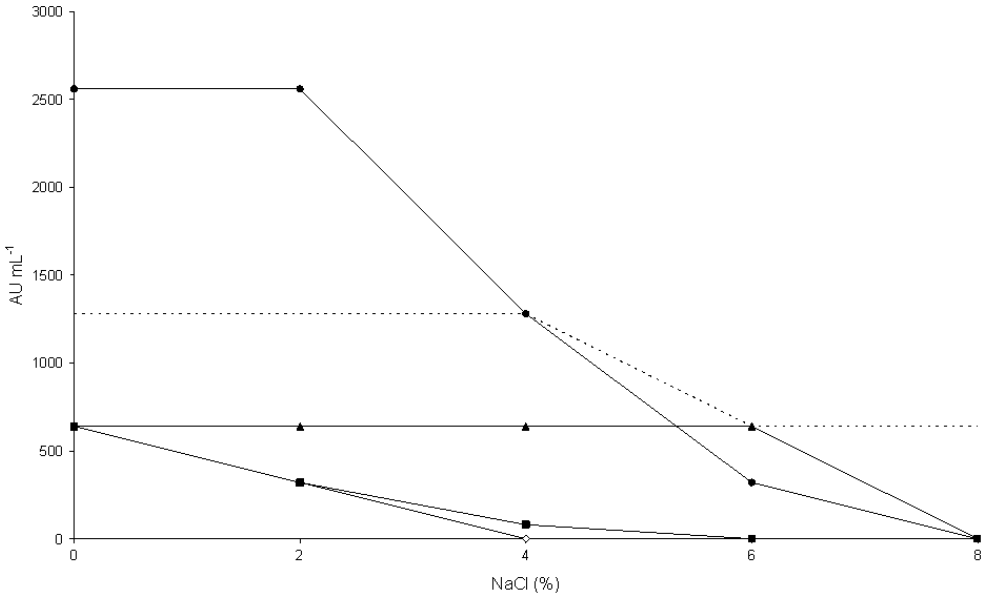

Fig. 2 - Effect of NaCl on BLIS production by *E. faecium* strains. Symbols: ◊, BLIS WGW7.2; ■, BLIS WGJ17.2; ▲, BLIS WGJ21.2; ●, BLIS WGJ28.1; ----, BLIS WGW33.2. The experiments were carried out in triplicate and the results shown are those confirmed in at least two independent measurements.

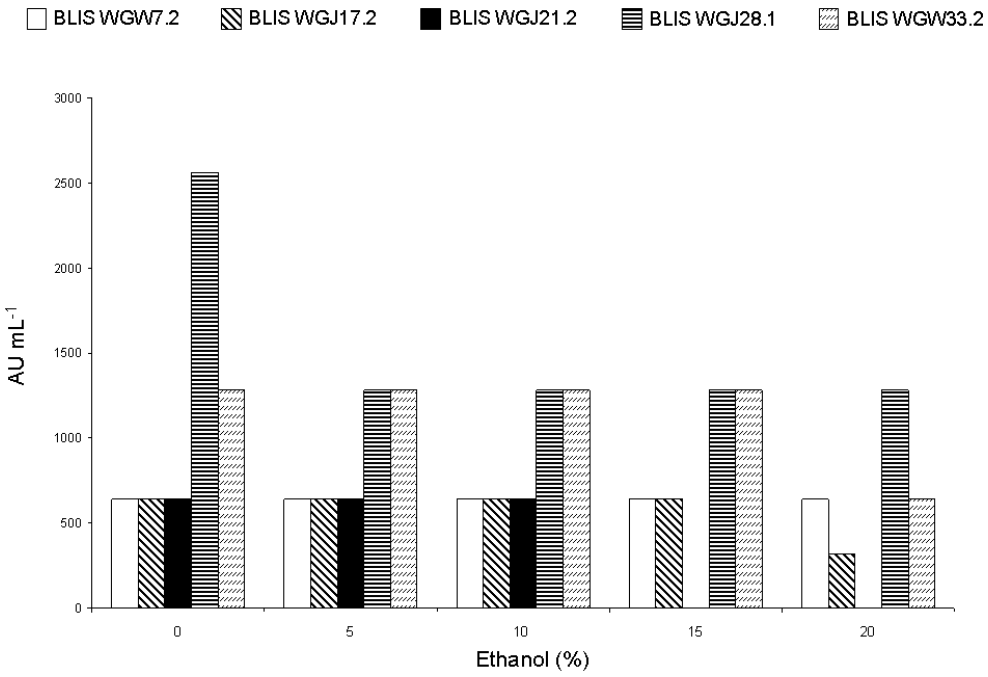

Fig. 3 - Effect of ethanol on BLIS production by *E. faecium* strains. The experiments were carried out in triplicate and the results shown are those confirmed in at least two independent measurements.

centrations increased and the strongest effect was recorded for strain WGJ17.2 and WGW7.2 (Fig. 2). The results with all the NaCl percentages tested were statistically significant ($P<0.05$). These results are in agreement with those of LEROY *et al.* (2003) who reported that salt stress decreased both cell growth and the specific bacteriocin production by *E. faecium* RZS C5. Those authors found that moderate levels of NaCl improved bacteriocin activity. They explained their findings by the fact that they increased the biomass concentration to the point at which bacteriocin production was shut off.

Ethanol did not influence BLIS activity from strain WGW7.2. In the 5-15% range (v/v) it had no effect on BLIS activity in strains WGJ17.2 and WGW33.2, while at 20% (v/v) it had a low impact (Fig. 3). BLIS activity of WGJ28.1 was slightly affected by 5% (v/v) ethanol and the same slight effect was observed at 20% (v/v). *E. faecium* WGJ21.2 was the only strain that was sensitive to the action of ethanol; BLIS activity ceased at a 15% (v/v) concentration. However, differences in ethanol BLIS production were only decisive at ethanol concentrations of 20% ($P=0.003$). On this basis, all the strains tested here, except *E. faecium* WGJ21.2, are more suitable for the biopreservation of foods and beverages that contain alcohol rather than brined vegetables.

No plasmids were extracted from *E. faecium* WGW7.2, WGJ17.2, WGJ21.2, WGJ28.1 or WGW33.2. Furthermore, treatment with acriflavine did not decrease BLIS activity. These results show that the genes encoding for the production of the five BLIS are located on the genomes.

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SHORT COMMUNICATION

IN VITRO PROBIOTIC CHARACTERISTICS OF *LACTOBACILLUS* STRAINS ISOLATED FROM FIORE SARDO CHEESE

CARATTERISTICHE PROBIOTICHE *IN VITRO*DI LATTOBACILLI ISOLATI DAL FORMAGGIO FIORE SARDO

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ABSTRACT

Fourteen *Lactobacillus* strains, isolated from artisanal Fiore Sardo cheese that were able to grow at pH 2.0 and in the presence of 0.3% bile, were evaluated for some functional characteristics relevant to their use as probiotic cultures. Most of the strains survived the conditions of the gastro-intestinal tract, and two also exhibited bile salt hydrolase activity. The levels of aggregation activity were similar in the strains tested. The most active strains also had high hydrophobicity levels. Most of the

RIASSUNTO

Sono state analizzate alcune caratteristiche funzionali di interesse probiotico in 14 ceppi di *Lactobacillus* (8 *L. plantarum* e sei *L. paracasei*) isolati dal formaggio Fiore Sardo, e risultati capaci di resistere a bassi valori di pH ed a concentrazioni fisiologiche di bile. Tra questi, 8 hanno ulteriormente confermato, *in vitro*, una buona capacità di sopravvivenza al passaggio nel tratto gastrointestinale, e due hanno mostrato capacità di idrolisi dei sali biliari. L'attività aggregante ha mostrato livelli simili

- Key words: acid and bile tolerance, antibiotic resistance, bile salt hydrolase, Fiore Sardo, *Lactobacillus*, probiotics -

strains showed antagonistic activity against the indicator strains *L. monocytogenes* ATCC 7644, *E. coli* ATCC 35150 and *Y. enterocolitica* ATCC 9610. High frequencies of antimicrobial resistance were observed towards aminoglycosides, glycopeptides, sulfamethoxazolo and trimethoprim. Six strains showed a number of *in vitro* properties that would make them suitable for use as adjunct cultures in the development of potentially probiotic cheese.

in entrambe le specie ed i ceppi più attivi hanno evidenziato anche i più alti livelli di idrofobicità. La maggior parte dei ceppi analizzati ha mostrato attività antagonistica nei confronti dei ceppi indicatori saggiati. Elevate frequenze di resistenza nei confronti degli antibiotici aminoglicosidi, glicopeptidi e sulfametossazolo/trimetoprim sono state osservate nei ceppi esaminati. In conclusione, 6 ceppi hanno mostrato di possedere, *in vitro*, alcune interessanti caratteristiche probiotiche. Questi ceppi potrebbero essere utilizzati come colture integrative nella preparazione di formaggi probiotici, tuttavia, ulteriori studi attraverso sperimentazioni *in vivo*, devono essere effettuati per confermarne la potenziale capacità di colonizzare il tratto intestinale.

INTRODUCTION

Lactic acid bacteria (LAB), normal components of the intestinal microflora in both humans and animals, have been associated with various health-promoting properties. Several food products or supplements containing viable LAB with probiotic properties, as well as pharmaceutical preparations, are commercially available. These products generally contain LAB strains, particularly from the genera *Lactobacillus*, *Enterococcus* and *Bifidobacterium*. These bacteria may have several therapeutic functions, including modulation of intestinal health and the immune system, improved lactose utilisation and anti-carcinogenic, anti-diarrhoeal and hypocholesterolaemic effects (FERNANDES *et al.*, 1987; SALMINEM *et al.*, 1998; PARODI, 1999).

Several aspects, including safety and physiological and functional properties, must be taken into account when selecting new probiotic strains. Probiotic strains should possess a Qualified Presumption of Safety (QPS) status and be able to survive through the gastro-intestinal tract. Furthermore, antagonistic activity toward potentially pathogenic microorganisms and immunomodulation, are the main requirements when selecting successful probiotic strains.

As a result of increasing awareness of the close interrelationship between health and diet, special attention is presently being given to functional properties of LAB that are associated with traditional fermented foods. These foods are produced with the help of spontaneous fermentation and in many cases the microorganisms involved have not been extensively characterised. Such products are a source of new microbial strains that have special physiological and functional properties that could have potential bio-

technological applications, e.g. as starter cultures and probiotic strains.

Lactobacilli are one of the major microbial groups involved in the fermentation of different types of food; they play an important role in preventing the growth of pathogenic and spoilage microorganisms. Mesophilic lactobacilli, such as *L. paracasei* and *L. plantarum*, constitute the majority of non-starter lactic acid bacteria (NSLAB) found in most ripened cheese varieties. They contribute to increasing the level of free amino acids, peptides and free fatty acids, which enhance flavour intensity and accelerate cheese ripening (LINCH *et al.*, 1999; MENÉNDEZ *et al.*, 2000).

In this study, 14 *Lactobacillus* strains, previously isolated from Fiore Sardo (an Italian Protected Designation of Origin cheese manufactured from raw ewes' milk without the addition of starter cultures) and able to grow at pH 2.0 and in the presence of 0.3% bile, were investigated for some functional characteristics relevant to their use as probiotic cultures such as *in vitro* resistance to gastro-intestinal conditions, aggregation ability, hydrophobic surface characteristics, antibacterial activity, bile salt deconjugation, susceptibility to antibiotics and production of biogenic amines.

MATERIALS AND METHODS

Bacterial cultures and growth conditions

Eight *Lactobacillus plantarum* and six *Lactobacillus paracasei* subsp. *paracasei* were tested. The strains, identified on the basis of their morphological, cultural and molecular characteristics (PISANO *et al.*, 2006), were maintained at -20°C in MRS broth (de Man Rogosa Sharpe, Oxoid, Basingstoke, UK) with 15% (v/v) glycerol (Microbiol, Cagliari, Italy) and propagated three times in MRS broth for activation prior to experimental use.

The following target bacteria, used for testing the antagonistic activity of the *Lactobacillus* strains, were purchased from the American Type Culture Collection (ATCC: Manassas, VA, USA) and the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ: Braunschweig, Germany): *Listeria monocytogenes* ATCC 7644, *Escherichia coli* ATCC 35150, *Yersinia enterocolitica* ATCC 9610, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, *Lactobacillus casei* DSM 20011 and *Lactobacillus sakei* subsp. *sakei* DSMZ 20017. All LAB strains were stored at -20°C in MRS broth with 15% (v/v) glycerol. *Listeria monocytogenes* ATCC 7644, *Escherichia coli* ATCC 35150, *Yersinia enterocolitica* ATCC 9610 and *Staphylococcus aureus* ATCC 25923 were maintained on nutrient broth (Microbiol) plus 20% (v/v) glycerol at -20°C. Before use, the strains were subcultured twice in MRS or nutrient broth.

In vitro resistance to gastro-intestinal conditions

The strains were inoculated to a final concentration of approximately $2.5x10^8$ cfu/mL in 10 mL of MRS broth acidified to pH 2.5 with 5M HCl, and incubated at 37°C with agitation to simulate peristalsis. After 1 h, 17.5 mL of synthetic duodenum juice (consisting of 6.4 g/L $NaHCO_3$, 0.239 g/L KCl, 1.28 g/L NaCl) adjusted to pH 7.4 with 5M HCl, and 4 mL of 10% (w/v) oxgall (Sigma, St. Louis, MO, USA) were added to the cell suspensions to simulate passage into the upper intestinal tract (FERNÁNDEZ *et al.*, 2003; VIZOSO PINTO *et al.*, 2006). After 0, 60, 120 and 180 min of incubation, the survival rate was determined by the plate method using MRS medium incubated anaerobically at 37°C for 48 h. The experiments were repeated twice and results are expressed as the mean log cfu/mL.

Bile salt hydrolase activity

Bile salt hydrolase (BSH) activity was screened by spotting in duplicate 10 μL of cultures grown overnight in MRS broth on the surface of MRS agar plates supplemented with 0.5% (w/v) sodium salt taurodeoxycholic acid (TDCA, Sigma) and 0.37 g/L of $CaCl_2$ (DASHKEVICZ and FEIGHNER, 1989). Plates were incubated in anaerobic conditions at 37°C for 72 h. There was BSH activity when deoxycholic acid precipitated in the agar medium below and around the colony. MRS agar plates without supplementation were used as controls.

Autoaggregation test

An autoaggregation test was performed as described by KOS *et al.* (2003). *Lactobacillus* strains were grown for 18 h at 37°C in MRS broth. The cells were harvested by centrifugation at 5,000 g for 15 min, washed twice and resuspended in an equal volume of phosphate buffered saline (PBS) with 10% (v/v) of filtered sterilised supernatant. Cell suspensions were mixed by vortexing for 10 s and autoaggregation was determined during a 3 h incubation period at room temperature. Every hour, 0.1 mL of the upper suspension was transferred to another tube with 3.9 mL of PBS and the optical density ($O.D_{600}$) was monitored. The autoaggregation is expressed as $1-(O.D_t/O.D_0)\times100$, where $O.D_t$ is the optical density at time 1, 2, 3 h; $O.D_0$ is the optical density at time 0 h.

Cell surface hydrophobicity

The level of hydrophobicity was determined by measuring the affinity of bacterial cells to a hydrophobic phase in a two-phase system (water:hexadecane, Sigma) according to KMET and LUCCHINI (1997). Cells were grown to a stationary phase in MRS broth at 37°C, centrifuged (5,000 g, 15 min), washed twice and resuspended in saline solution to 0.5-0.6 $O.D_{560}$ units. Equal volumes of bacterial suspension and hexadecane were mixed together and vortexed for 60 s. The suspensions were left at room temperature for 1 h to allow the two phases to separate. The aqueous phase was carefully removed and the $O.D_{560}$ was measured. The percentage of hydrophobicity was evaluated as the percentage of reduction in the optical density of the aqueous phase.

Antimicrobial activity assay

An agar spot test and the well diffusion assay, described by SHILLINGER and LÜCKE (1989), were used with slight modifications to detect antimicrobial activity. In the first case, overnight-cultures of lactobacilli were spotted (5 μL) onto the surface of MRS agar (1.2% (w/v) agar -0.2% (w/v) glucose) plates, which were then incubated anaerobically for 24 h at 37°C. The indicator strains were inoculated into 7 mL of soft agar medium (MRS or nutrient broth containing 0.7% w/v agar) to a final concentration of approximately 10^7 cfu/mL. The soft media were poured on the plates which were incubated for 24 h at the optimal growth temperature and atmosphere for the indicator strains. Inhibition was scored positive in the presence of a detectable clear zone around the colony of the producer strain.

The strains with the best inhibitory activity in the agar spot-test were further tested using the well diffusion assay. Briefly, a 200 μL aliquot of an overnight culture of the indicator bacteria was inoculated into 20 mL of appropriate soft agar medium and poured into the Petri dishes. Thereafter, wells (6 mm diameter) were cut into the agar and 100 μL aliquots of cell-free supernatant of the potential producer strains, collected by centrifugation (10,000 g, 15 min), were placed into each well. In order to eliminate the inhibitory effect of lactic acid

and/or H_2O_2, the supernatants were adjusted to pH 6.5 with 5M NaOH, treated with catalase (1 mg/mL, Sigma), then filtered through a 0.45 μm pore-size cellulose acetate filter (Millipore, Bedford MA, USA). The plates were refrigerated for 4 h to allow the radial diffusion of the compounds contained in the supernatant prior to incubation for 24 h at the optimal growth temperature for the indicator strains. The antimicrobial activity was indicated by the presence of inhibition zones.

Biogenic amines

The method of BOVER-CID and HOLZAPFEL (1999) was used to screen *Lactobacillus* strains for the production of biogenic amines. Briefly, the test strains were subcultured twice at 24 h intervals in MRS broth containing 1% of each precursor amino acid: tyrosine disodium salt, L-histidine monohydrochloride, L-ornithine monohydrochloride and L-lysine monohydrocloride (Sigma), and 0.005% pyridoxal-5-phosphate (Sigma) as a codecarboxylase factor (BOVER-CID and HOLZAPFEL, 1999). All strains were then streaked in duplicate on decarboxylase medium plates each containing only one of the above-mentioned amino acids and bromocresol purple as pH indicator and incubated for 4 days in anaerobic conditions at 37°C. Decarboxylase medium without amino acids was used as control. A colour change from brown to purple in the medium indicated an increase in pH and was considered a positive result.

Antibiotic resistance test

The disc diffusion method of BAUER *et al.* (1966) was used to determine the susceptibility of the strains to the following antiobiotics (Oxoid): ampicillin, piperacillin, oxacillin, penicillin G, vancomycin, teicoplanin, cephalothin (inhibitors of cell wall synthesis), streptomycin, tetracycline, gentamicin, kanamycin, erythromycin, chloramphenicol, clindamycin, fosfomycin, quinupristin/dalfopristin (inhibitors of protein synthesis), ciprofloxacin, rifampicin, norfloxacin, co-trimoxazole and nitrofurantoin (inhibitors of nucleic acids). A suspension from fresh overnight cultures, with a density of McFarland 0.5 in buffered saline was plated on Muller-Hinton (Microbiol) agar plates. Antibiotic discs were dispensed onto the plates. After incubation at 37°C for 24 h in anaerobiosis, the diameters of the bacterial free-zone were measured and results are expressed in terms of resistance according to the interpretative criteria issued by the Clinical and Laboratory Standards Institute (CLSI), formerly the National Committee for Clinical Laboratory Standards (NCCLS, 2000).

RESULTS

In vitro resistance to gastro-intestinal conditions

The strains were examined for their ability to survive passage in the upper intestinal tract, using a simulated stomach-duodenum passage (SSDP) (Fig. 1). The number of living cells of the *L. plantarum* DBS 273, DBS 324, DBS 384, DBS 385, DBS 387 and *L. paracasei* subsp. *paracasei* DBS 396 strains did not decrease during simulated gastric intestinal conditions. After 180 min, the number of surviving cells was about $1x10^8$ cfu/mL.

The *L. plantarum* DBS 330, 389 and *L. paracasei* subsp. *paracasei* DBS 328, DBS 370, DBS 386 and DBS 394 strains survived satisfactorily during simulated stomach passage although they were affected by exposure to the toxic effects of bile salts. The viable counts in *L. plantarum* DBS 115 and *L. paracasei* DBS 371 strains decreased significantly after 120 min of exposure to bile salts.

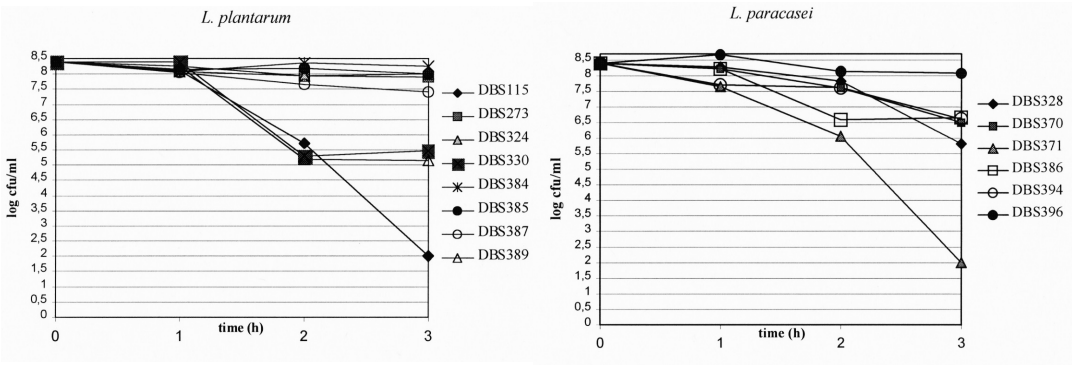

Fig. 1 - Survival of *Lactobacillus* strains to artificial gastric juice (pH 2.5) after 1 h and to artificial duodenal secretion (pH 7.4) after 2 and 3 h.

BSH activity

All of the strains tested were able to grow in MRS-TDCA plates but only two *L. plantarum* strains (DBS 324 and DBS 385) showed BSH activity on MRS-TDCA plates (data not shown).

Aggregation properties and surface characteristics

Aggregation assay was performed by measuring the sedimentation rate of the strains over a 3 h period. Levels of autoaggregation activity, expressed as the percentage reduction in absorbance after 3 h, varied between 38 and 47.8% in all strains tested (Fig. 2).

The surface hydrophobicity of the strains was determined by measuring bacterial adhesion to n-hexadecane. The most active strains were *L. plantarum* DBS 385, DBS 387, and *L. paracasei* subsp. *paracasei* DBS 371, DBS 394 and DBS 396. In general, the strains with the highest levels of autoaggregation were also the most hydrophobic.

Antimicrobial activity

The agar spot test method was used to screen the strains for antagonis-

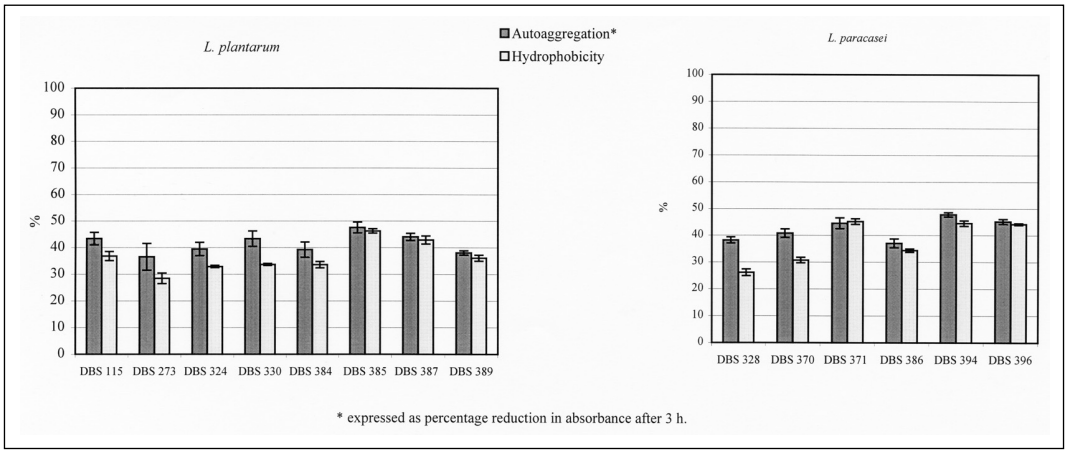

Fig. 2 - Autoaggregation ability and hydrophobicity of *Lactobacillus* strains. Error bars represent standard deviations of the mean values from three experiments.

Table 1 - Antagonistic activity of *Lactobacillus* strains toward indicator bacteria.

Strain	Inhibition of indicator bacteria*						
	L. monocytogenes ATCC 7644	*E. coli* ATCC 35150	*Y. enterocolitica* ATCC 9610	*L. sakei* DSM 20017	*L. casei* DSM 20011	*S. aureus* ATCC 25923	*E. faecalis* ATCC 29212
L. plantarum							
DBS115	+++	+++	+++	+	+	+	++
DBS273	+++	+++	+++	+	+	+	+
DBS324	+	+++	+++	-	-	+	+
DBS330	+++	+++	+++	-	+	+	+
DBS384	+++	+++	+++	+	+	+	+
DBS385	+++	+++	+++	+	+	+	+
DBS387	++	+++	+++	-	-	+	+
DBS389	+++	++	++	-	-	+	+
L. paracasei							
DBS328	+++	+++	+++	+	+	+	+++
DBS370	++	++	+++	+	+	+	+
DBS371	++	++	++	-	-	+	+
DBS386	+++	+++	+++	+	+	++	++
DBS 394	+++	+++	+++	-	-	+	++
DBS396	++	+++	+++	+	+	+	+

* +++ 8 mm of inhibition and above, ++ between 4 and 7 mm of inhibition, + between 1 and 3 mm of inhibition, - no inhibition.

tic activity. As shown in Table 1, all strains displayed inhibition of growth towards two or more indicator bacteria, with the strongest activity against the food-borne pathogens *L. monocytogenes*, *E. coli* O157:H7 and *Y. enterocolitica*. Neutralised supernatants of the strains having the best inhibitory activity were tested by the agar well diffusion assay against all the indicator strains, to investigate the presence of bacteriocin-like compounds; no inhibitory activity was observed (data not shown).

Biogenic amine production

None of the strains tested in this study decarboxylated lysine, histidine, ornithine or tyrosine (data not shown).

Antibiotic resistance

The antibiotic resistance data for *Lactobacillus* strains are reported in Table 2. Within the group of antimicrobial agents that inhibit cell wall synthesis, all strains were resistant to oxacillin and vancomycin and susceptible to piperacillin. All *L. plantarum* and most *L. paracasei* subsp. *paracasei* strains were resistant to teicoplanin. Most of the *L. plantarum* strains were also resistant to penicillin G and cephalothin. All strains were susceptible to chloramphenicol, erythromycin and quinupristin/dalfopristin, and resistant to fosfomycin. High levels of resistance towards the aminoglycosides gentamycin, streptomycin and kanamycin were observed,

Table 2 - Antibiotic resistance of *Lactobacillus* strains isolated from Fiore Sardo cheese.

Strain	Inhibitors of cell wall synthesis							Inhibitors of protein synthesis									Inhibitors of nucleic acids				
	Ampicillin	Penicillin G	Oxacillin	Piperacillin	Vancomycin	Teicoplanin	Cephalothin	Streptomycin	Tetracycline	Gentamycin	Kanamycin	Chloramphenicol	Erythromycin	Clindamycin	Fosfomycin	Quinupristin/dalfopristin	Ciprofloxacin	Rifampicin	Norfloxacin	Co-trimoxazole	Nitrofurantoin
L. plantarum																					
DBS115	S	S	R	S	R	R	R	S	S	R	R	S	S	S	R	S	R	S	R	R	S
DBS273	S	R	R	S	R	R	R	R	S	S	R	S	S	S	R	S	R	S	R	R	R
DBS324	S	R	R	S	R	R	S	R	S	R	R	S	S	R	R	S	R	R	R	R	S
DBS330	R	R	R	S	R	R	R	R	R	S	R	S	S	S	R	S	R	R	R	R	R
DBS384	S	R	R	S	R	R	R	S	R	R	R	S	S	S	R	S	R	S	R	R	R
DBS385	R	R	R	S	R	R	R	R	R	S	R	S	S	S	R	S	R	S	R	R	S
DBS387	S	R	R	S	R	R	S	R	S	R	R	S	S	S	R	S	R	S	R	R	R
DBS389	S	R	R	S	R	R	R	R	R	S	R	S	S	S	R	S	R	S	R	R	R
L. paracasei																					
DBS328	R	S	R	S	R	R	S	R	S	R	R	S	S	R	R	S	R	S	R	R	S
DBS370	R	S	R	S	R	R	S	R	S	S	R	S	S	S	R	S	S	S	S	R	S
DBS371	S	S	R	S	R	R	S	R	S	R	R	S	S	S	R	S	S	S	S	R	S
DBS386	S	S	R	S	R	R	S	R	S	S	R	S	S	S	R	S	R	S	R	R	S
DBS394	S	R	R	S	R	S	R	R	S	R	R	S	S	S	R	S	R	S	R	R	S
DBS396	S	R	R	S	R	S	R	R	R	S	R	S	S	S	R	S	S	S	R	R	S

R, resistant; S, susceptible.

in strains of both species. Regarding the antibiotics that inhibit the synthesis of nucleic acids, all of the strains were resistant to co-trimoxazole; all of the *L. plantarum* strains were also resistant to ciprofloxacin and norfloxacin.

DISCUSSION

A probiotic bacterium must be able to survive passage through the acidic environment of the stomach and resist being digested by bile salts. To date most studies have focused on the autochthonous microflora associated with the gastro-intestinal (G-I) tract of some mammals (JIN *et al.*, 1998; FERNÁNDEZ *et al.*, 2003; RODRÍGUEZ *et al.*, 2003). Much more attention has recently been given to the potential probiotic traits of isolates associated with naturally fermented foods such as dairy products (COEURET *et al.*, 2004; VIZOSO PINTO *et al.*, 2006). These could be used as carriers, because their high fat content may protect the strains from the stressful conditions of the G-I tract.

The 14 *Lactobacillus* strains analysed in this study were isolated from different Fiore Sardo cheese samples and were initially characterised as *L. plantarum* or *L. paracasei* subsp. *paracasei* by using biochemical and metabolic parameters and species-specific PCR (data not shown). These strains were resistant at pH 2.0 and the subsequent stress of 0.3% bile (data not shown); the *in vitro*

ability of these strains to pass and survive the G-I tract was further investigated by using the SSDP test, which gives predictive values for the assumed survival of digested LAB in the human stomach and duodenum. Many of the strains tested survived through the upper intestinal tract. The susceptibility to the higher bile concentration used could explain the low number of some strains after 180 min at 37°C.

BSH activity is frequently observed in specific bacterial groups such as *Lactobacillus*, *Bifidobacterium* and *Enterococcus* isolated from the G-I tract, where it serves as a detoxification reaction to protect bacteria from the toxicity of conjugated bile acids (DE SMET *et al.*, 1995). It is hypothesised that deconjugation of bile acids may help reduce serum cholesterol by increasing the demand for cholesterol for the *de novo* synthesis of bile acids, because free bile salts are more likely to be excreted from the G-I tract than conjugated bile salts (GILLILAND, 1990; DE SMET *et al.*, 1994). NGUYEN *et al.* (2007) identified and characterised a strain of *L. plantarum* with cholesterol-lowering activities. TANAKA *et al.* (1999) suggested that BSH expression in LAB is strongly correlated with the natural habitat; species that live in the human intestine have a high incidence of BSH-active strains. In contrast, species from other habitats such as milk or vegetables have a low incidence of BSH-active strains. Of the 14 strains tested, only two exhibited BSH activity, while all of the strains grew in the presence of conjugated bile salts after 24 h of incubation. These results are in line with other authors who found *Lactobacillus* strains that were resistant to physiologically conjugated bile salt concentrations but did not express BSH activity (HALLER *et al.*, 2001; MOSER and SAVAGE, 2001; RODRÍGUEZ *et al.*, 2003).

Another important property that increases the potential of bacteria to survive and persist in the G-I tract is their ability to adhere to tissues, which may affect the colonisation of the human and animal environments. Autoaggregation ability and bacterial surface hydrophobicity are thought to be linked to the ability to adhere to epithelial cells of the G-I tract and could be used as a preliminary screening to identify potentially adherent bacteria (GRANATO *et al.*, 1999; DEL RE *et al.*, 2000). In the present study, aggregation activity seems to be a homogeneous characteristic in both *Lactobacillus* species analysed. Higher levels of autoaggregation have been observed for obligately homofermentative lactobacilli strains including *L. crispatus*, *L. acidophilus* and *L. gasseri* (RENIERO *et al.*, 1992; KOS *et al.*, 2003; MARCOTTE *et al.*, 2004). All of the *Lactobacillus* strains tested had hydrophobicity values that were similar to some commercial *L. paracasei* strains used in probiotic yoghurt-like products (SCHILLINGER *et al.*, 2005). The fact that the strains that had the most aggregation activity had the highest hydrophobicity levels is in agreement with other authors who reported a correlation between hydrophobicity and autoaggregation in *Lactobacillus* strains (KMET and LUCCHINI, 1997; MARCOTTE *et al.*, 2004).

LAB produce many different substances with antimicrobial activity (SERVIN, 2004), including the major metabolic end products such as various organic acids, hydrogen peroxide, ethanol and bacteriocins. In this study, several *Lactobacillus* strains demonstrated antagonistic activity against some indicator strains. The strongest inhibitory activity was towards the food-borne pathogens *L. monocytogenes*, *E. coli* O157:H7 and *Y. enterocolitica*. When the pH value of the supernatants was adjusted to 6.5 and catalase was added, no inhibitory effect was observed. Presumably this indicates that either acid production or hydrogen peroxide were responsible for the observed antimicrobial effect.

Since probiotic strains must be safe for human consumption, the European

Food Safety Authority (EFSA) recently granted QPS status to a number of *Lactobacillus* species (including *L. plantarum* and *L. paracasei*), based on their long history of safe use in foods. The QPS concept, similar to the GRAS system in the United States, would allow microorganisms, for which there are no special safety concerns, to enter the market without extensive testing requirements (EFSA, 2007).

The formation of biogenic amines is a concern in terms of food safety and quality. Biogenic amines are produced by LAB through amino acid decarboxylation during the process of fermentation of foods and beverages. In cheese, this has often been linked to non-starter lactic acid bacteria and *Enterobacteriaceae* (JOOSTEN and NORTHOLT, 1987). BOVER-CID and HOLZAPFEL (1999) reported that the ability to produce biogenic amines in a synthetic medium may be strain-dependent rather than being related to a specific species. In the present screening, none of the strains displayed amino acid decarboxylase activity.

The *Lactobacillus* strains were assayed for their resistance to 21 antibiotics using a disc diffusion method on Mueller-Hinton agar plates under anaerobic conditions. High frequencies of resistance were observed against most of the antibiotics tested; this suggests that the strains tested would not be affected by therapy using these antibiotics and might help maintain the natural balance of intestinal microflora during antibiotic treatment. According to the EFSA the presence of acquired antibiotic resistance is an important safety criterion for determining the QPS status of a strain (EFSA, 2007). Resistance to some antibiotics such as aminoglycosides, quinolones and glycopeptides appears to be intrinsic for lactobacilli (KLEIN *et al.*, 2000; DANIELSEN and WIND, 2003; TEMMERMAN *et al.*, 2003). Vancomycin resistance has been well documented in lactobacilli and is attributed to the synthesis of modified cell wall peptidoglycan precursors that end in a depsipeptide d-alanine-d-lactate instead of the dipeptide d-alanine-d-alanine which is the target for vancomycin activity (HANDWERGER *et al.*, 1994). KLEIN *et al.* (2000) showed that the glycopeptide-resistance in *Lactobacillus* strains is not of the transmissible type. The high frequency of tetracycline resistance observed in *L. plantarum* strains is important, given that plasmid-mediated tetracycline resistance has been demonstrated in this species (DANIELSEN, 2002). Further studies should focus on the location and potential transferability of tetracycline resistance determinants.

CONCLUSION

The present study identified 14 *Lactobacillus* strains isolated from artisanal Fiore Sardo cheese possessing several interesting *in vitro* properties that may be essential for probiotic function. In particular, five *L. plantarum* strains and one *L. paracasei* subsp. *paracasei* strain survived under conditions similar to those encountered in the G-I tract. Two strains, DBS 324 and DBS 385, also displayed BSH activity. While these *in vitro* properties cannot fully predict the functionality of the strains in the human body, they are helpful in selecting *Lactobacillus* strains to be used as probiotics. These strains may be potential probiotics that could be used as adjunct cultures in the development of potentially probiotic cheese. *In vivo* studies are needed to determine their persistence in the human gut, after ingestion.

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SHORT COMMUNICATION

RESIDUAL LEVELS OF Cd, Pb AND As IN NON SMOKED AND SMOKED "PROVOLA" CHEESE FROM CALABRIA (ITALY)

LIVELLI RESIDUALI DI Cd, Pb E As IN "PROVOLE" AFFUMICATE E NON DELLA CALABRIA (ITALIA)

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ABSTRACT

Residual levels of Cd, Pb and As in "provola" cheese from Calabria, non-smoked, smoked with natural methods and commercial buffered smoke, were evaluated. The highest concentrations of Cd and As were found in naturally smoked "provola" cheese, while the highest levels of Pb were recorded in buffered smoked samples. These results, also expressed according to provisional tolerable daily intake (PTDI) by FAO/WHO, have showed that non-smoked, naturally smoked and buff-

RIASSUNTO

Sono stati valutati i livelli residuali di Cd, Pb e As in "provole" della Calabria, non affumicate, affumicate con metodo naturale e fumo liquido commerciale. I risultati documentano concentrazioni più elevate di Cd e As in "provole" affumicate con metodo naturale mentre per il Pb i livelli più elevati sono stati riscontrati nei campioni affumicati con fumo liquido. Questi risultati, espressi anche come PTDI secondo la FAO/WHO, hanno mostrato che le provole non affumicate, affumicate con metodo naturale e

- Key words: buffered smoke, Calabria, heavy metals, "provola" cheese -

ered smoked "provola" cheeses do not contribute significantly to heavy metal intake in the human diet. The use of controlled commercial buffered smoke, however, is a safer technique for the smoking process of "provola" cheese as far as the amounts of toxic metals, except for Pb, is concerned.

con fumo liquido non danno un contributo significativo all'assorbimento dei metalli nell'uomo con la dieta. L'uso di fumo liquido commerciale, pertanto, può essere considerata una tecnica più sicura per l'affumicatura delle provole relativamente alla presenza di metalli tossici, fatta eccezione per i Pb.

INTRODUCTION

Heavy metals are ubiquitous elements present in nature that have become important toxic contaminants due to human activities that have significantly increased their presence in the environment. They enter the food chain easily and can cause toxic effects in humans and animals (CERUTTI, 1999; CASARETT and DOULL, 2000).

Milk and dairy products are important components of the human diet but their consumption can contribute significantly to heavy metal intake (Cd, As and Pb). LICATA *et al.* (2004) and NACCARI *et al.* (2006) investigated the presence of toxic metals (As, Cd and Pb) in bovine milk samples from different farms in Calabria and Sicily. The Pb concentrations in milk samples from Sicily were higher than those found in samples from Calabria and exceeded the MRLs (Maximum Residue Limits) established by EC Regulation n.2001/466.

Cheese is an important food in Mediterranean diet, rich in protein, lipids, calcium and vitamins (SCOTT, 1981; FOX *et al.*, 1996). The consumption of smoked cheese is very common because it is a typical product with organoleptic properties and methods of smoking in various geographical areas.

The process used for smoking can also contribute to the presence of trace metals in cheese. The traditional smoking process is done under non-controlled conditions regarding temperature, humidity and type of wood for combustion (oat straw, beech shavings, shrubs, heath, laurel and myrtle, poplar shavings, olive and orange wood, etc.) and the smoke generated comes into direct contact with the cheese during processing. Commercial buffered smoke, introduced recently in European countries for smoking foods, has the advantages of allowing product uniformity, reproducibility of the characteristics of the end-product, cleanliness of application and controlled amounts of toxic compounds in smoke flavourings before being added to the food (SIMON *et al.*, 2005). In Italy, the Legislative Decree 107/1992 (1999) on smoke flavourings for food established the composition of buffered smoke and fixed the maximum permissible levels in smoking aroma at 1 mg/kg for Cd, 3 mg/kg for As and 10 mg/kg for Pb, as previously specified by Council Directives 88/3888/CEE (1988).

The FAO/WHO has set limits for heavy metal intake based on body weight: for an adult of 60 kg a "provisional tolerable daily intake" (PTDI) of 60 μg/day, 128 μg/day and 214 μg/day for Cd, As and Pb, respectively (Joint FAO/WHO 1999).

Other researchers have studied the presence of heavy metals in dairy products (GAMBELLI *et al.*, 1999; IMPARATO *et al.*, 1999; ORAK *et al.*, 2005; MENDIL, 2006) but today there are no data con-

cerning the presence of traces of heavy metals in smoked cheese. To evaluate the role of smoking on heavy metal concentration, the aim of this study was to determine the residual levels of As, Cd and Pb in non-smoked, naturally and buffered smoked "provola" cheese manufactured in Calabria.

MATERIALS AND METHODS

Sampling

"Provola" is a fresh cow milk cheese, prepared using full-cream milk, coagulated with kid or lamb rennet paste. It is a raw, stretched curd cheese, moulded manually or with the use of special spinners, salted for immersion in a brine bath or smoked and matured for about 24 hours at room temperature. It is a sweet, white, round cheese; when it is exposed to the smoking process the external part darkens, acquires a dark yellow colour and aromatic flavour.

The present investigation was conducted on 45 samples of "provola" cheese (15 non-smoked, 15 smoked with traditional methods and 15 with commercial buffered smoke), a local product of Calabria, randomly collected from different farms.

Sample preparation and analyses

The samples were subjected to homogenization with a stainless steel blender, kept in PET containers and frozen at -20°C until analysis. Aliquots of 0.5 g were taken from each sample and submitted to digestion overnight with 2 mL of HNO_3 (70% v/v). The samples were mineralized in a microwave oven (model MDS-2100, CEM corporation, Matthews, NC, USA; power 950±50 W at 100%) and Teflon™ PFA reactors, equipped with a pressure regulation system (by means of a vessel acting as a sensor), in two different steps according to the program illustrated in Table 1. Analytical blanks were prepared in a similar manner without samples to check possible contamination during analysis. After the first step, 1.8 mL of H_2O_2 (30% v/v) were added to the samples, which then underwent the second step of mineralization. The samples and blank solutions were brought to a volume of 10 mL with ultrapure water. All the glassware used during the analysis was treated with a dilute solution of HNO_3 (0.1% v/v) to prevent contamination.

The concentrations of various heavy metals were determined by two methods of atomic absorption spectroscopy (NACCARI *et al.*, 2006; AOAC, 1990).

1) Analysis by graphite furnace atomic absorption spectrometry (GF-AAS) for Cd and Pb, carried out with an AA Varian model 220/Zeeman spectrophotometer (Varian Australia, Mulgrade, Victoria, Australia).

2) Analysis by a hot vapor generation technique (HG-AAS) for As was carried out with a VGA 77 adapted to a Varian AA-475 spectrophotometer (Varian Australia, Mulgrade, Victoria, Australia). 10 M HCl was used for the acid channel and $NaBH_4$ (0.6% v/v) and KI (10% v/v) in NaOH (0.5% v/v) were used for the reducing channel.

The quantification of all elements was performed using the external standard method.

The experimental conditions adopted for each metal are reported in Table 1; the data obtained for each metal is the mean value of four determinations.

Reagents

Nitric acid (70%), hydrogen peroxide (30%) and ultrapure water for analysis of trace metals were provided by J.T. Backer (Mallinckrodt Backer, Milan, Italy). Standard stock solutions (1,000 ppm) of Pb, Cd and As were provided by MERCK (Darmstand, Germany). The potassium iodide, sodium borohydride and sodium hydroxide used were of analytical reagent grade (Sigma-Aldrich Milan, Italy).

Table 1 - Working conditions adopted for determining Cd, Pb and As levels.

GF-AAS	Wavelength (nm)	Slit (nm)	Stage	Temperature (°C)	Time (s)	Gas Flow (L/min)
Cd	228.8	0.5	1	85	5	3.0
			2	95	40	3.0
			3	120	10	3.0
			4	250	5	3.0
			5	250	1	3.0
			6	250	2	0.0
			7	1,800	0.8	0.0
			8	1,800	2	0.0
			9	2,000	0.1	3.0
			10	2,000	2	3.0
Pb	283.3	0.5	1	85	5	3.0
			2	95	40	3.0
			3	120	10	3.0
			4	600	5	3.0
			5	600	2.1	3.0
			6	600	2.1	0.0
			7	2,200	1	0.0
			8	2,200	2	0.0
			9	2,400	2	3.0

HG-AAS	Wavelength (nm)	Slit (nm)	Flame composition	Reductant channel	Acid channel
As	193.7	0.5	Air - acetylene	0.6% $NaBH_4$ +10% KI in 0.5% NaOH	10 M HCl

Calibration method

To check the linearity, standard mixtures at four concentration levels (1-2.5-5-7.5 and 10 μg kg^{-1} for As and Cd; 10-20-30-40 and 50 μg kg^{-1} for Pb) were analyzed. The above-mentioned mixtures were obtained from a matrix solution of 1 mg kg^{-1}. A calibration curve was constructed by plotting the mean (n=4) of abs vs standard concentration in ng/mL.

The accuracy and repeatability of the method were assessed by performing a spike-and-recovery test. The sample blanks were fortified with the metals before digestion. Recovery was measured using fortified samples (n=3 replicates) each at four concentrations (75, 100, 125 and 150 μg kg^{-1} for As; 15, 20, 25 and 30 μg kg^{-1} for Cd; 225, 300, 375 and 450 μg kg^{-1} for Pb). Spike recoveries were repeated three times and the results are expressed as an average percentage of recovery. Recovery values, always above 87% for As, 100% for Cd and 98.6% for Pb, showed the accuracy of analysis.

To determine precision, 200 μL of standard solution were added to the negative control (non-smoked cheese samples) to obtain final concentrations of 75, 100, 125 and 150 μg kg^{-1} for As; 15, 20, 25 and 30 μg kg^{-1} for Cd; 225, 300, 375 and 450 μg kg^{-1} for Pb. The fortified samples were mineralized and analyzed as described earlier.

In order to determine the detection limits of an element, two solutions were prepared: the concentration of the first solution was close to the detection limits, the concentration of the second solution was double the detection limit concentration. The test was repeated 12 times.

The detection limits were in accordance with the following formula:

$$\text{Detection limit in ng/mL} = \frac{3 \times \text{standard deviation (control)} \times \text{lower concentration (standard)}}{\text{average absorbance of standard signal at lower concentration used}}$$

Good laboratory practice (GLP) was applied throughout and procedural blanks were also analysed. The specificity was confirmed by analysis of blank samples; for all elements the blank concentrations were lower than the respective detection limits.

The parameters of the calibration method are reported in Table 2.

Statistical analysis

Data are expressed as mean ± S.D. of at least four determinations. They were statistically analyzed by one-way analysis of variance (ANOVA) and the Student- Newman-Keults test for unpaired data (TALLARIRA RT and MURRAY RB, 1986). A value of $P<0.05$ was considered statistically significant.

RESULTS AND CONCLUSIONS

The residual levels of toxic metals Cd, Pb and As evaluated in non-smoked, naturally smoked and buffered smoked "provola" cheese samples from Calabria are reported in Table 3. The results are reported as mean ± standard deviation, range values and percentage of positive sample for each metal.

The results show the presence of trace heavy metals in all "provola" cheese samples analyzed with the concentrations of Pb and As being higher than those of Cd. The comparative analysis of these metals showed that the residual levels of Cd in naturally smoked "provola" cheese (2.78±1.27 μg kg^{-1}) are statistically significant ($P<0.05$) and higher than in buffered smoked (1.27±0.64 μg kg^{-1}) and non-smoked samples (0.93±0.40 μg kg^{-1}). The Pb concentrations in naturally smoked "provola" cheese (34.20±18.09 μg kg^{-1}) were statistically significant ($P<0.05$) and higher than in non-smoked samples (11.56±6.1 μg kg^{-1}) but less than in buffered smoked samples (108.64±23.39 μg kg^{-1}). The Pb levels in buffered smoked samples (108.64±23.39 μg kg^{-1}) were also higher than non buffered samples (11.56±6.1 μg kg^{-1}) and were statistically significant ($P<0.01$).

The As values were higher in natural-

Table 2 - Precision, average of recovery, linearity of the analytical procedure and detection limits.

	μg/kg added	Recovery %	Linearity	sr (%)[a]	Absolute detection limit[b]
Cd	15-30	100	0.998	2.68	0.018
Pb	225-450	98.6	0.989	0.2545	0.601
As	75-150	87	0.966	0.2828	0.15

[a]sr (%): precision of five independent determinations, expressed as relative standard deviation.
[b]Absolute detection limits (ng/L) =[3 x S.D. (control) x Conc. (stand.)]/average absorbance of standard.
S.D. (control) = standard deviation of control.
Conc. (stand.) = lowest concentration of standard used (ng/mL).
Average absorbance of standard = Abs signal at lowest concentrations.

Table 3 - Residual levels of Cd, Pb and As expressed as mean (M) ± standard deviation (S.D.) μg kg^{-1} of individual measurements) in 45 "provola" cheese samples from Calabria.

Samples	Cd $M \pm SD$	Pb Range	As positive samples %	$M \pm SD$	Range	positive samples %	$M \pm SD$	Range	positive samples %
non-smoked "Provola"cheese (n = 15)	0.93±0.40	0.43-1.40	14 (93%)	11.56±6.05	8.27-15.08	13 (86%)	29.90±14.94	12.96-56.34	15 (100%)
naturally smoked "Provola"cheese (n = 15)	2.78±1.27&*	1.35-4.40	14 (93%)	34.20±18.09&*	13.20-55.65	15 (100%)	51.73±24.08*	25.80-90.27	15 (100%)
buffered smoked "Provola"cheese (n = 15)	1.27±0.64	0.55-2.12	15 (100%)	108.64±23.39§	81.2-132.93	13 (86%)	34.54±10.35	18.51-49.17	14 (93%)

*P<0.05 vs non-smoked;
&P<0.05 vs buffered smoked;
§P<0.01 vs non-smoked.

ly smoked "provola" cheese samples (51.73±24.08 μg kg^{-1}) than in buffered smoked (34.54±10.35 μg kg^{-1}) and in non-smoked (26.90±14.94 μg kg^{-1}) samples and were statistically significant (P<0.05).

In non-smoked "provola" cheese samples the high levels of As and Pb than Cd were similar to those reported by various authors for Kasar cheese (YUZBASI *et al.*, 2003), fresh white curd cheese (DEMIROZU-ERDINC and SALDAMLI, 2000), and "ricotta" and "caciotta" dairy products of southern Italy (ANASTASIO *et al.*, 2006).

The concentrations of Cd and As in natural smoked "provola" cheese were the highest, while the concentrations found in buffered smoked "provola" cheese were similar to those of non-smoked samples. There are no data in the literature concerning the presence of trace heavy metals in smoked cheese.

The Pb (108.64±23.39 μg kg^{-1}), Cd (1.27±0.64 μg kg^{-1}) and As (34.54±10.35 μg kg^{-1}) values in buffered smoked "provola" cheese samples are acceptable because the Legislative Decree 107/1992 (1992) and Council Directives 88/3888/CEE (1988) have set the following maximum levels in smoking aroma at 10 mg kg^{-1}, 1 mg kg^{-1} and 3 mg kg^{-1} for Pb, Cd and As, respectively.

The higher Pb level in buffered smoked "provola" cheese can be correlated to higher concentrations that are also found in milk samples, as demonstrated in previous analyses (LICATA *et al.*, 2004; NACCARI *et al.*, 2006). It can be hypothesized that the Pb level is the sum of the Pb concentration present in the milk used to produce the cheese and the residual levels present in buffered smoke flavourings used during the smoking process of "provola" cheese.

Table 4 - Residual levels of Cd, Pb and As in 45 "provola" cheese samples from Calabria. Data are expressed as mean (M) ± standard deviation (S.D) μg kg^{-1} of individual measurements and as % of provisional tolerable daily intake (PTDI), calculated for 100 g of cheese eaten (Joint, FAO/WHO 1999).

Samples	Cd		Pb		As	
	μg kg^{-1}	% PTDI (60 μg/die)	μg kg^{-1}	% PTDI (214μg/die)	μg kg^{-1}	% PTDI (128μg/die)
non smoked "Provola "cheese	0.93±0.40	0.15	11.56±6.05	0.540	26.90±14.94	2.10
naturally smoked "Provola" cheese	2.78±1.27&*	0.46	34.20±18.09&*	1.598	51.73±24.08*	4.04
buffered smoked "Provola" cheese	1.27±0.64	0.21	108.64±23.39§	5.07	34.54±10.35	2.71

*P<0.05 vs non-smoked;
&P<0.05 vs buffered smoked;
§P<0.01 vs non- smoked.

The FAO/WHO has set the daily limit for the intake of heavy metals based on body weight and the Joint Expert Committee on Food Additives (JEFCA, Joint FAO/WHO, 1999) has indicated that the "provisional tolerable daily intake" (PTDI) for an adult weighing 60 kg is 60 μg/day, 214 μg/day and 128 μg/day for Cd, Pb and As, respectively. According to JECFA, the residual levels of heavy metals reported in this study correspond to 0.15, 0.46 and 0.21% of PTDI for Cd, 0.54, 1.59 and 5.07% of PTDI for Pb, 2.10, 4.04 and 2.69% of PTDI for As, in non-smoked, naturally smoked and buffered smoked "provola" cheese samples, respectively (Table 4), based on 100 g of cheese eaten.

In conclusion, the residual levels of Cd, Pb and As in cheese are probably due to the concentration of trace elements during manufacturing (types of milk and rennet used, whether the mold is used or not and the degree of ripening) and the optional smoking process of the cheese.

The higher levels of heavy metals in naturally smoked cheese demonstrate that this non-controlled smoking process which uses natural wood materials for combustion results in a more significant concentration of heavy metals in "provola" cheese compared with commercial buffered smoke, except for Pb. Standardization of these traditional smoking procedures is advisable.

The residual levels of Cd, Pb and As found in non-smoked, naturally smoked and buffered smoked "provola" cheese samples from Calabria are, however, only a small percentage of PTDI allowed by the Joint Expert Committee on Food Additives. Therefore consumption of non-smoked, naturally smoked and buffered smoked "provola" cheeses does not contribute significantly to heavy metal intake in the human diet.

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SHORT COMMUNICATION

EFFECTS OF CENTRIFUGATION ON MALOLACTIC FERMENTATION IN RED WINE

EFFETTI DELLA CENTRIFUGAZIONE SULLA FERMENTAZIONE MALOLATTICA IN VINO ROSSO

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ABSTRACT

The centrifugation of wine is a practice used by industrial wineries for rapid wine clarification. The effect of centrifugation on spontaneous malolactic fermentation (MLF) in red wine has been evaluated. Red wine was centrifuged after devatting, by using an industrial disc centrifuge, at flow rates of 3,500, 2,000, 1,500 and 1,000 L/h and at a constant rotational speed of 11,000 rpm. Centrifuged wines had a lower nitrogen and

RIASSUNTO

La centrifugazione è una pratica utilizzata dalle cantine industriali per chiarificare rapidamente il vino. Sono stati valutati gli effetti della centrifugazione sulla fermentazione malolattica (FML) spontanea in vino rosso. Dopo svinatura, il vino è stato centrifugato, mediante una centrifuga industriale a dischi, alle portate di 3.500, 2.000, 1.500 e 1.000 L/h con rotazione costante a 11.000 rpm. I vini cen-

- Key words: biogenic amines, malolactic fermentation, *Oenococcus oeni*, wine centrifugation -

proanthocyanidin concentration than the control (not centrifuged) wine. Malic acid consumption was favoured in wine centrifuged at 3,500 L/h but was delayed at 1,500 and 1,000 L/h. Indigenous lactic acid bacteria decreased, at most, by 2 $\log_{10}$ cfu/mL. Moreover, after MLF, the total content of biogenic amines decreased in centrifuged wines. Putrescine was the most abundant amine detected (4.84-7.34 mg/L), while low concentrations of histamine were found (0.82-1.05 mg/L).

trifugati presentavano un minore contenuto in azoto e di proantocianidine. Il consumo di acido malico era favorito nel vino centrifugato a 3.500 L/h e ritardato a 1.500 e 1.000 L/h. I batteri lattici indigeni sono diminuiti massimo 2 $\log_{10}$ ufc/mL. Dopo la FML il contenuto totale di ammine biogene nei vini centrifugati era minore rispetto al controllo. La putrescina è risultata la più abbondante ammina rilevata (4,84-7,34 mg/L), mentre il contenuto in istamina è risultato basso (0,82-1,05 mg/L).

INTRODUCTION

In winemaking centrifugation is used to clarify musts and wines (RIBÉREAU-GAYON *et al.*, 2006). Must centrifugation allows the rapid removal of suspended grape solids. In wineries centrifugation is widely used in different phases of vinification: at devatting to rapidly reduce wine turbidity, in fining treatments, performed by adding organic (e.g. proteins) (MAURY *et al.*, 2003) or inorganic substances (e.g. bentonite) (MUHLACK *et al.*, 2006) and at bottling to facilitate the filtration process. Moreover, centrifugation can be used to arrest alcoholic fermentation (AF) in the production of sweet wines.

Centrifugation performed after wine devatting results in a decrease in the yeast and bacterial populations. Nevertheless, to effectively remove bacteria, high rotational speeds (15,000-20,000 rpm) are necessary (RIBÉREAU-GAYON *et al.*, 2006). Moreover, high speed centrifugation could reduce important wine bacteria growth factors, such as nitrogen sources, vitamins and fatty acids, that would affect malolactic fermentation (MLF) kinetics. Excessive must clarification could inhibit fermentation activities (DELFINI *et al.*, 1993). FERRANDO *et al.* (1998) observed that must centrifugation, compared to other clarification techniques such as vacuum filtration, does not seem to negatively influence the AF kinetics and wine quality.

Since the centrifugation of wine after AF has many advantages, this practice has become increasingly more widespread in industrial wine production (RIBÉREAU-GAYON *et al.*, 2006). Consequently, an investigation of the effects of this kind of clarification on MLF is of importance for the science of winemaking. To the best of our knowledge no data are available on the effects of centrifugation of wine on MLF, that is either induced by indigenous strains or by inoculation with selected strains.

The aim of this study was to evaluate the main effects of centrifugation on the chemical and microbiological composition of wine. Industrial disc centrifugation was applied at different flow rates after wine devatting. The impact of centrifugation on indigenous lactic acid bacteria was evaluated by monitoring MLF.

MATERIALS AND METHODS

Vinification and centrifugation

Fermentation of grapes for the production of Valpolicella wine (Italy) was

carried out in a tank by *Saccharomyces cerevisiae* Lalvin D80 (Lallemand Inc., Montreal, Canada). AF was monitored by analysing sugar consumption and ethanol production. After AF, the wine was analysed and divided into four parts to be centrifuged at different flow rates. A disc centrifuge (model SE 161 EIX, Seital, Vicenza, Italy), consisting of a bowl rotating at 11,000 rpm with a maximum capacity of 6,000 L/h was used. Wine samples were centrifuged at flow rates of 3,500, 2,000, 1,500 and 1,000 L/h, and then transferred to tanks containing 100 L of each sample, called 3,500, 2,000, 1,500 and 1,000 wine. Spontaneous MLF was monitored by measuring the malic acid depletion and determining the LAB concentration. Trials were carried out in a wine cellar where the temperature ranged from 18°-20°C.

Wine analysis

Alcohol, pH, total acidity, residual sugars and total dry extract content were determined by the official OIV methods. Organic acids were quantified using enzyme kits (La Roche, Basel, Switzerland). Total polyphenols, total anthocyanins, proanthocyanidins, wine colour density and hue were quantified spectrophotometrically according to DI STEFANO *et al.* (1989). Nitrogen quantification was carried out by mineralisation according to the method of HACH *et al.* (1985). Wine turbidity, expressed in Nephelometric Turbidity Units (NTU), was measured before and after the centrifugation treatment, using a Hach 2100N turbidimeter (Hach Company, Loveland, Colorado) calibrated with Formazin turbidity standard 4000 NTU. The presence of eight biogenic amines (BA) (histamine, cadaverine, putrescine, phenylethylalanine, amylamine, isobutylamine, methylamine and isopropylamine) was determined in wine after MLF by HPLC according to TORREA and ANCIN (2001).

Microbiological analysis and species identification

For the enumeration of yeasts and LAB, wine samples were plated in duplicate on WL nutrient agar medium (Oxoid Ltd., London, UK) and MRS (Oxoid) plus 10% tomato juice (MRS-tj) with 0.02% cycloheximide. For the yeast counts, plates were incubated aerobically at 28°C for three days, while for LAB counts, an Anaerocult A kit (Merck, Darmstadt, Germany) was used and plates were incubated anaerobically for seven days.

A total of 30 isolates (six isolates per trial) were randomly taken from the plates for identification. Yeasts were classified on the basis of colony morphology according to PALLMAN *et al.* (2001). In order to identify the LAB, a preliminary classification was carried out based on morphological and biochemical tests such as gram staining, and catalase and sugar fermentation. Rod-shaped isolates were identified by sugar fermentation (FUGELSANG and EDWARDS, 2007), while coccal-shaped ones were shown to belong to *Oenococcus oeni* through species-specific PCR according to ZAPPAROLI *et al.* (1998).

Statistical treatment of the data

Microvinifications were carried out in triplicate and wine analyses were performed for each independent trial. Data were statistically treated applying the T-test and significant differences ($p<0.05$) are reported.

RESULTS

After centrifugation the wine turbidity values, proanthocyanidin concentrations and total nitrogen content decreased significantly (Table 1). The proanthocyanidin concentrations were not statistically significant in the 3,500 wine but decreased significantly in the other samples with respect to the control. Total

Table 1 - Composition of non-centrifuged wine (control) and wines centrifuged at various flow rates.

		Control	Centrifugation flow rates (L/h)			
			3,500	2,000	1,500	1,000
Turbidity	NDT	1075.3±8.6 a[1]	12.8±0.2 b	4.9±0.1 c	3.0±0.0 d	2.4±0.0 e
pH		3.30±0.01	3.31±0.01	3.32±0.01	3.33±0.00	3.33±0.01
Ethanol	% vol.	10.93±0.05	10.89±0.06	10.92±0.02	10.90±0.03	10.95±0.03
Residual sugars	g/L	5.41±0.12	5.35±0.09	5.45±0.15	5.23±0.10	5.42±0.11
Total acidity	g tartaric acid/L	6.20±0.03	6.18±0.02	6.25±0.03	6.18±0.02	6.21±0.02
Acetic acid	g/L	0.14±0.01	0.13±0.01	0.14±0.01	0.13±0.00	0.13±0.01
Malic acid	g/L	1.59±0.05	1.61±0.04	1.63±0.03	1.60±0.04	1.60±0.03
L-lactic acid	g/L	0.05±0.01	0.05±0.00	0.04±0.01	0.06±0.01	0.05±0.01
Total polyphenols	mg gallic acid/L	1187±79	1226±44	1231±8	1223±8	1254±8
Total anthocyanins	mg malvidin-3 glucoside/L	360±6	353±7	364±15	353±5	354±3
Proanthocyanidins	mg cyanidin/L	3152±25 a	3182±18 a	3036±22 b	3036±23 b	2988±17 b
Colour density		1.01±0.01 a	1.11±0.11 a	1.12±0.07a	1.19±0.01 b	1.17±0.01 b
Hue		0.45±0.00	0.45±0.00	0.44±0.00	0.45±0.00	0.45±0.00
Total nitrogen	mg/L	154±11 a	109±3 b	100±2 c	98±2 c	89±2 d

[1] Values followed by different letters within a row are significantly different at p<0.05.

nitrogen content was reduced by about 30% in wine centrifuged at 3,500 L/h flow rate, by 36% in wine centrifuged at 2,000 and 1,500 L/h flow rates and by 42% in wine centrifuged at 1,000 L/h flow rate in comparison with the control wine.

After AF but before centrifugation the microbial populations were constituted by yeasts and LAB. Yeasts, grown on WL medium, were identified as *Saccharomyces* spp., on the basis of colony and cell morphology. Their ascription to *S. cerevisiae* is highly probable because a commercial strain of this species was inoculated in the must to carry out AF. The isolates from MRS-tj plates were identified as *L. plantarum* by sugar fermentation pattern for rods and as *O. oeni* by species-specific PCR (data not shown). The estimated concentrations of *Lactobacillus* spp. and *O. oeni*. were $6.0x10^3$ and $2.0x10^3$ cfu/mL, respectively. The degree to which the centrifugation of wine the removed the yeasts and LAB depended on the flow rate (Fig. 1). The yeast cells were removed more effectively than bacteria. Using low flow rates, yeasts were almost completely eliminated (a reduction of about 7 $\log_{10}$ cfu/mL for the 1000 wine compared with the control). About 2 $\log_{10}$ cfu/mL of *O. oeni* cells were removed. The application of a flow rate of 3,500 L/h did not significantly reduce the *O. oeni* cell concentration, but decreased that of *Lactobacillus* spp. In the 1,500 and 1,000 wines the *Lactobacillus* spp. population disappeared.

Fig. 2 shows the kinetics of MLF in all of the wines. The onset and completion of malic acid depletion occurred earlier in the 3500 wine

with respect to the other wines. Wine centrifugation at lower flow rates either did not produce any differences (2,000 L/h) or prolonged MLF (1,500 and 1,000 L/h) with respect to the control (Fig. 2A). Moreover, the different initial cell concentrations in the wines produced affected the growth kinetics of the bacterial population (Fig. 2B).

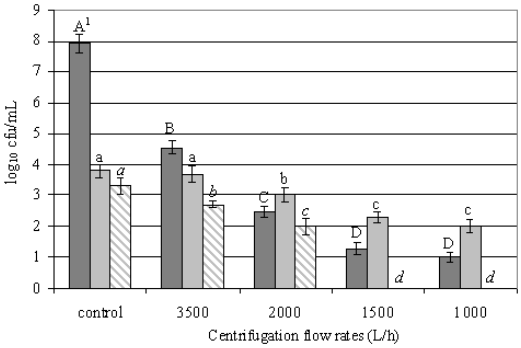

Fig. 1 - Concentration of *Saccharomyces cerevisiae* (dark grey columns), *Oenococcus oeni* (light grey columns) and *Lactobacillus* spp. (crossed columns) determined in non-centrifuged wine (control) and wines centrifuged at various flow rates.
[1]Values followed by different letters (capital for *S. cerevisiae*, small for *O. oeni* and italics for *Lactobacillus* spp.) on each column are significantly different at $p<0.05$.

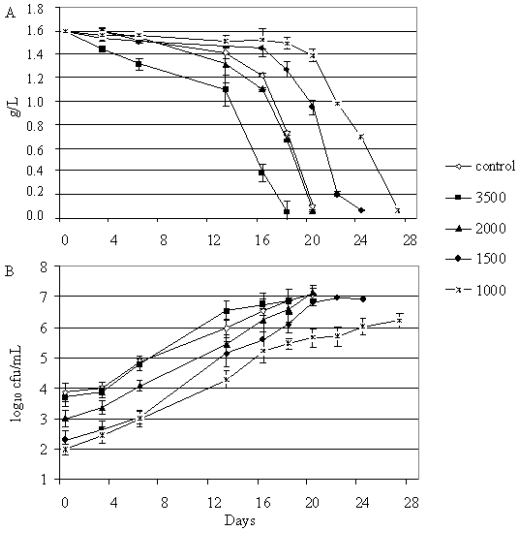

Fig. 2 - Malic acid (g/L) (A) and lactic acid bacteria concentration ($\log_{10}$ cfu/mL) (B) measured in non-centrifuged wine (control) and wines centrifuged at the flow rates of 3,500, 2,000, 1,500 and 1,000 L/h.

In all of the wines, MLF was carried out prevalently by *O. oeni*. In the control, 3,500 and 2,000 wines the *Lactobacillus* spp. concentrations remained low ($<10^4$ cfu/mL) in correspondence to the maximum rate of malic acid consumption. In these wines the contribution of the residual *Lactobacillus* spp. cells to MLF seems to have been negligible. The results were not correlated with the reduction in the number of indigenous malolactic bacteria cells obtained by the different centrifugations (Fig. 1). Even though the bacteria concentration in the 3,500 wine was similar to that of the control, the growth was faster and MLF was completed sooner than in the non-centrifuged wine.

Since biogenic amines are undesirable substances that are generally produced in wine by decarboxylase activity of LAB, some important amines were quantified in all of the wines after MLF (Table 2). The amylamine and putrescine concentrations decreased in all of the centrifuged wines with respect to the control. Histamine was present at low concentrations in all of the wines, but the content was significantly higher in the 1,000 wine with respect to the others.

DISCUSSION

The most relevant consequence of centrifugation on the chemical composition of wine was the variation in the nitrogen content. Studies conducted on the production of extracellular protease by *O. oeni* elucidated the mechanisms involved in the utilization of the nitrogen source during MLF in wine (REMIZE *et al.*, 2005). Since the proteins/peptides, produced by yeasts, can inhibit *O. oeni* growth (COMITINI *et al.*, 2005; OSBORNE

Table 2 - Biogenic amine content (mg/L) determined on non-centrifuged wine (control) and wines centrifuged at various flow rates.

	Control	Centrifugation flow rates (L/h)			
		3,500	2,000	1,500	1,000
Amylamine	0.45±0.04 a[1]	0.34±0.01 b	0.32±0.01 b	0.36±0.02 b	0.37±0.02 b
Cadaverine	0.00	0.00	0.00	0.00	0.00
Phenylethylamine	0.09±0.01	0.10±0.00	0.09±0.01	0.11±0.01	0.08±0.01
Isobuthylamine	0.00	0.00	0.00	0.00	0.00
Histamine	0.86±0.06 a	0.82±0.02 a	0.91±0.01 a	0.85±0.01 a	1.05±0.05 b
Methylamine	0.00	0.00	0.00	0.00	0.00
n-Propylamine	0.00	0.00	0.00	0.00	0.00
Putrescine	7.34±0.03 a	5.31±0.07 b	5.25±0.10 b	5.47±0.11 b	4.84±0.11 c
Tyramine	0.29±0.02	0.30±0.02	0.27±0.02	0.31±0.02	0.30±0.01
Total	9.01±0.02 a	6.87±0.08 b	6.84±0.10 b	7.10±0.13 b	6.64±0.07 c

[1] Values followed by different letters within a row are significantly different at $p<0.05$.

and EDWARDS, 2007), a depletion in wine proteins could affect the growth of bacteria in centrifuged wine. Similarly, centrifugation could deplete other nutritional factors needed for LAB. GUERRINI *et al.* (2002), studying the effects of oleic acid on *O. oeni*, stressed that MLF could be negatively affected if oleic acid is lacking in the wine due to clarification.

The effects of removing the cells by centrifugation, partially explain the results of the MLF kinetics. The reduction of the initial cell concentration by centrifugation mainly affects the onset of MLF. Because low flow rates (<1,500 L/h) cause a marginal reduction of the bacteria concentration they could be used to delay the population growth. On the other hand, high flow rates (>2,000 L/h) seem to either have no effects or favour spontaneous MLF. Besides cell concentration, the effectiveness of centrifugation on the removal of the cells also depends on cell shape and size as well as the type of association (single, in pairs or in chains). Since it has been demonstrated that the cell size of wine microorganisms is related to their physiological state (MILLET and LONVAUD-FUNEL, 2000), gentle centrifugation at the devatting stage could selectively modify the bacterial composition, thereby affecting MLF.

Wine storage in the presence of lees appears to contribute to the increase of BA in wine (MARQUES *et al.*, 2008). Therefore, after AF, the amount of residual yeast lees should be a source of BA formation. The puterscine content in the control and centrifuged wines could be explained by different accumulations of ornithine due to contact with the lees during MLF. *O. oeni* strains can contribute significantly to the BA content in wine, particularly by the accumulation of putrescine (MANGANI *et al.*, 2005). Clarification by centrifugation before MLF may affect the production of BA.

In conclusion, this study demonstrates that wine centrifugation, carried out at the devatting stage, has a significant impact on the microbial component of wine. This process could affect the competition between indigenous and selected malolactic bacterial strains when wine is inoculated with a starter. Further investigations are needed to better understand the effect that centrifugation has on the availability of nutritional factors and on the removal of substances that inhibit the growth of bacteria.

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SHORT COMMUNICATION

ANTIOXIDANT AND ANTIBACTERIAL ACTIVITIES OF *PORTULACA OLERACEA* L. GROWN WILD IN TURKEY

ATTIVITÀ ANTIBATTERICA ED ANTIOSSIDANTE DELLA *PORTULACA OLERACEA* L. SELVATICA DELLA TURCHIA

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ABSTRACT

The *in vitro* antioxidant and antibacterial activities of methanol extracts from leaves of wild purslane (*Portulaca oleracea* L.) from Turkey were investigated. The plant extracts were screened for possible antioxidant activity by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and β-carotene-linoleic acid assays. The MeOH extracts exhibited high levels of free radical scavenging activity (IC_{50}=54.33±1.26 μg/mL). The purslane leaves also showed high antioxidant activity (91.15%) compared to

RIASSUNTO

Sono state studiate le proprietà antiossidanti ed antibatteriche dell'estratto metanolico delle foglie di *Portulaca oleracea* L. selvatica della Turchia. Gli estratti della pianta sono stati analizzati per quanto riguarda l'attività antiossidante utilizzando i saggi del 2,2-difenil-1-picrildrazile (DPPH) e del β-carotene-acido linoleico. Gli estratti metanolici presentavano elevati livelli di attività nei confronti dei radicali liberi (IC_{50}=54.33±1.26 μg/mL). Anche le foglie presentavano alta attività

- Key words: antibacterial activity, antioxidant activity, fatty acids, *Portulaca oleracea* L. -

synthetic antioxidants BHA (98.16%) and BHT (96.66%). Although the antioxidant activity of the leaf extracts was lower than the BHA and BHT, the difference was not statistically significant, $P<0.05$. The amount of total phenolics was 17.88 μgGAE/mg DW. Total lipids in purslane leaves were 5.83%. Linolenic acid was the dominant fatty acid (56.33%) followed by linoleic (14.01%), palmitic (9.72%), oleic (8.83%), myristic (5.04%) and stearic (4.36%) acids. The methanol extracts from the leaves showed antibacterial activities against *Bacillus subtilis, Pseudomonas syringae* pv. *tomato, Vibrio cholerae* and *Yersinia pseudotuberculosis.* None of the water extracts showed antibacterial activity against the microorganisms studied.

antiossidante (91.15%) rispetto all'acido linoleico, confrontabile con antiossidanti sintetici come il BHA (98.16%) ed il BHT (96.66%). Il contenuto in composti fenolici totali era di 17.88 μg GAE/mg DW. I lipidi totali nelle foglie di *Portulaca oleracea* L. era 5.83%. L'acido linolenico era l'acido grasso dominante (56.33%) seguito dal linoleico (14.01%), palmitico (9.72%), oleico (8.83%), miristico (5.04%) e stearico (4.36%). Gli estratti metanolici delle foglie mostravano attività antibatterica contro il *Bacillus subtilis,* la *Pseudomonas syringae* pv. *tomato,* il *Vibrio cholorae* e la *Yersinia pseudotuberculosis.* Nessuno degli estratti acquosi mostrava invece attività antibatterica sui microrganismi considerati.

INTRODUCTION

Portulaca oleracea L., commonly known as purslane, is an herbaceous weed. The plant grows wild and/or cultivated throughout much of the world. The World Health Organization has indicated that it is one of the most widely used medicinal plants, calling it a "global panacea". It is ranked eighth among the most commonly distributed plants in the world; it can be eaten both fresh and dried (BAYTOP, 2000; DWECK, 2001; SAMY *et al.*, 2004). It is palatable and has a mild flavor. The tender stems and leaves can be eaten raw, cooked, or pickled (SIMOPOULOS and SALEM, 1986; SIMOPOULOS *et al.*, 1992). The edible stems and leaves have a slightly acidic, salty taste, similar to spinach. It is available commercially in both ornamental and culinary cultivars and is widely used as a potherb in Mediterranean, central European and Asian countries. The aerial parts of the plant are used to alleviate pain and swelling and as an antiseptic (CHAN *et al.*, 2000). A recent report indicated that an extract of *P. oleracea* accelerates wound healing and is used to treat indomethacine and phenylbutazone-induced ulcers (RASHED *et al.*, 2003).

Interest in cultivating *P. oleracea* as a food crop has been stimulated because it contains many bioactive compounds including ascorbic acid, proteins, fatty acids, flavonoids, vitamin E and beta carotene (EZEKWE *et al.*, 1999). The quantity of these compounds in *P. oleracea* varies with the growing conditions (e.g., planting date, soil quality, fertilization) and the age of the plant (LIM and QUAH, 2007).

Wild edible *P. oleracea* plants are found in most parts of Anatolia in Turkey particularly near cultivated and arid seaside areas. In Anatolia the people consume only wild *P. oleracea* as food and use it for medicinal purposes due to economical, traditional and geographical rea-

sons. In recent years it has also been widely cultivated in western Turkey for use in salads and as food additives (OZBUCAK *et al.*, 2005). *P. oleracea* has been used to treat the following ailments in humans: alterative, antiseptic, ardor, diuretic, emollient, scurvy and sedative (BAYTOP, 2000).

Recent studies have focused on the health functions of medicinal plants which have antioxidant, antimicrobial and antimutagen properties (NAKAMURA *et al.*, 2003). Dietary intake of antioxidant compounds is important for health (DUH *et al.*, 1999). Synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) which are commonly used in processed foods have been reported to have some negative side effects (ITO *et al.*, 1983). Therefore there has been increased interest in finding plants with medicinal properties and high antioxidant capacities. Medicinal plants are the main source of new pharmaceuticals and healthcare products. Plant products have a potential use for food preservation; the antioxidant capacity of plant extracts are strongly related to their phenolic compound content (LIM and QUAH, 2007).

Another health function of medicinal plants is due to their essential fatty acid composition. Humans cannot synthesize these compounds and must obtain them through the diet. Essential fatty acids are required for the formation of healthy cell membranes and the proper development and functioning of the brain and nervous system (PAWLOSKY *et al.*, 1996; SIMOPOULOS and SALEM, 1986).

For centuries, indigenous plants have been used in herbal medicine to cure various diseases (COWAN, 1999). Currently, there is growing interest in the use of natural antibacterial compounds. Plant extracts from herbs and spices are used to preserve foods because they have characteristic flavor and sometimes show antibacterial activity (SMID and GORRIS, 1999). The acceptance of traditional medicine as an alternative form for health care and the development of microbial resistance to the currently available antibiotics have led researchers to investigate the antibacterial activity of medicinal plants (OZTURK and ERCISLI, 2007).

Information is lacking on the nutritional value of *P. oleracea* accessions from different regions of the world. There have been a few studies on the bioactive compounds of *P. oleracea*, but little information has been published regarding its nutrient composition. Cultivated plants were usually used in these studies. The antibacterial properties of wild *P. oleracea* have not been reported in detail. The aim of the present study was to determine the total phenolic, fatty acid and mineral content of *P. oleracea* as well as its antioxidant and antibacterial activities.

MATERIALS AND METHODS

Mature (14-true-leaf stage) wild *P. oleracea* plants were harvested manually from ten locations having similar soil types in Erzurum in the Eastern Anatolia region of Turkey. At least 30 individual plants were pooled to form a single sample after collection. They were put in a portable refrigerator and transported to the laboratory (2-3 h). All plant material was authenticated. Samples were divided into two groups and the first group of leaves (fresh) was used for the ascorbic acid analysis. The second group was dried in an oven at 45°C for 3 days and ground into a fine powder with a mortar and pestle and kept at room temperature prior to extraction. The dried samples were put into new plastic bags and stored in a dessicator for a maximum of 3 days until the antioxidant activity, total phenolics, fatty acids and antibacterial activity analyses were carried out.

The sample (about 100 g) was extract-

ed in a Soxhlet extractor with methanol (MeOH) at 60°C for 6 h. The extract was then filtered and concentrated *in vacuo* at 45°C. Finally, the extracts were lyophilized and kept in the dark at +4°C until tested.

All reagents were obtained from Sigma Chemical Co. (St Louis, MO, USA) and were analytical grade.

Total lipids of the dried material were extracted according to the method of GUIL-GUERRERO and RODRIGUEZ-GARCIA (1999). The fatty acid composition of *P. oleracea* leaves was analyzed according to a previous method (ANON, 2000). The protein and mineral and vitamin C contents of air-dried *P. oleracea* leaves were determined according to the methods of AOAC (1984).

The hydrogen atom-electron donating ability of the corresponding extracts and some pure compounds were measured on the basis of the % bleaching of a purple methanol solution of DPPH. In this spectrophotometric assay, the stable radical diphenylpicrylhydrazyl (DPPH) was used as reagent (Sigma-Aldrich, St. Lois, MO, USA) (BURITS and BUCAR, 2000). Fifty μL of various concentrations of the extracts in methanol were added to 5 mL of a 0.004% methanol solution of DPPH. After a 30 min incubation period at room temperature, the absorbance was read against a blank at 517 nm. Inhibition of the free radical DPPH was calculated in percent (*I*%) as follows:

$I\,\% = (A_{blank} - A_{sample}/A_{blank}) \times 100$ where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. The extract concentration that provided 50% inhibition (*IC*50) was calculated from the graph with inhibition plotted against extract concentration. Tests were carried out in triplicate; butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), curcumine and ascorbic acid were used as positive controls.

In the β-carotene-linoleic acid assay, the antioxidant capacity is determined by measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation (BARRIERE *et al.*, 2001).

A stock solution of β-carotene/linoleic acid (Sigma-Aldrich) was prepared as follows: 0.5 mg of β-carotene was dissolved in 1 mL of chloroform (HPLC grade), then 25 μL of linoleic acid and 200 mg of Tween 40 (Merck, Darmstadt, Germany) were added. The chloroform was subsequently evaporated using a vacuum evaporator. Then, 100 mL of distilled water saturated with oxygen (30 min at 100 mL/min) were added with vigorous shaking. Aliquots (2.5 mL) of this reaction mixture were transferred to test tubes, and 350 μL portions of the extracts (2 g/L in ethanol) were added before incubating for 48 h at room temperature. The same procedure was repeated with BHA and BHT at the same concentrations and a blank containing only 350 μL of ethanol. After the incubation period, the absorbance of the mixtures was measured at 490 nm. Antioxidant capacities of the samples were compared with those of BHA, BHT and the blank.

Total phenolic constituents of *P. oleracea* leaves were determined by the method of SLINKARD and SINGLETON (1977) using Folin-Ciocalteu reagent and gallic acid as standard.

To determine the mineral composition, samples were burned with nitric acid and perchloric acid solutions on the hot plate at 200°C. The absorbance of the extract was then measured with an Atomic Absorbance Spectrophotometer (Perkin Elmer 2380, Waltham/MA, USA). The mineral content was calculated from a standard curve for each element. The phosphorus content in the extract was analyzed by determining the absorbance of the yellow-colored solution, obtained from the Barton reaction, using a spectrophotometer (Thermo, Nicolet 100, UV, London, UK) at 680 nm wavelength, and

comparing the results to the standard curve (JAMES, 1995).

To test antibacterial activity the following microorganisms were used: *Pseudomonas syringae* pv. *syringae*, *Pseudomonas syringae* pv. *tomato*, *Bacillus subtilis*, *Bacillus cereus* GC subgroup A, *Yersinia enterocolita*, *Vibrio cholerae* non 01, *Corynebacterium diphteriae*, *Yersinia frederiksenii*, *Yersinia pseudotuberculosis*, *Salmonella typhimurium* GC subgroup A, *Serratia liquefaciens*, *Pseudomonas corrugate*, *Xanthomonas compestris compestris*, *Agrobacterium tumafaciens* and *Pseudomonas aeruginosa*. The bacteria, maintained on Nutrient Agar (Merck, Darmstadt, Germany), were supplied by the Microbiology Laboratory of the Agricultural Faculty of Ataturk University, Erzurum, Turkey. The identity of the bacteria used in this study was confirmed by the Microbial Identification System in the Biotechnology Application and Research Center at Ataturk University.

The agar well-diffusion method was used to determine the antibacterial activity. The extracts were filter-sterilized using a 0.45 μm membrane filter. Each microorganism was suspended in sterile saline solution and diluted to 10^8 colony-forming units (cfu)/mL of bacteria spread on nutrient agar (NA) medium. The wells (8 mm in diameter) were cut from the agar and 0.06 mL of extract solution was put into them. After incubation for 24 h at 37°C, all plates were examined for any growth inhibition zones and the diameter of these zones was measured (mm). The minimal inhibition concentration (MIC) values were also studied. The inocula of bacteria were prepared from 12 h broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. *P. oleracea* extracts were first dissolved in 0.5% dimethylsulfoxide (DMSO) prepared at a concentration of 500 μg/mL and then serial two-fold dilutions were made in a concentration range from 3.90 to 500 μg/mL in 10 mL sterile test tubes containing nutrient broth. MIC values of the extracts against bacterial strains were determined based on a micro-well dilution method (OZTURK and ERCISLI, 2007). The 96-well plates were prepared by putting 95 μL of nutrient broth and 5 μL of the inoculum into each well. One-hundred μL of the extracts initially prepared at the concentration of 500 μg /mL were added to the first well. Then, 100 μL from the serial dilutions were transferred to six consecutive wells. The last well, containing 195 μL of nutrient broth without compound and 5 μL of the inoculum on each strip was used as the negative control. The final volume in each well was 200 μL. Maxipime (Bristol-Myers Squibb, Cincinnati, OH, USA) at a concentration range of 500-7.8 μg /mL was prepared in nutrient broth and used as standard drug for the positive control. The contents of each well were mixed on a plate shaker at 300 rpm for 20 s and then incubated at appropriate temperatures for 24 h. Microbial growth was determined by absorbance at 600 nm using the ELx 800 universal microplate reader (Biotek Instrument Inc., Highland Park, VT, USA) and confirmed by plating 5 μL samples from clear wells on nutrient agar medium. The extract tested in this study was screened twice against each organism. The MIC of each extract was taken as the lowest concentration that showed no growth (OZTURK and ERCISLI, 2007).

The experiment was a completely randomized design with four replications. Data were subjected to analysis of variance (ANOVA) and means were separated by Duncan's multiple range test at $P<0.05$ significant level.

RESULTS AND DISCUSSION

The moisture and protein contents of *P. oleracea* leaves were 84% and 33 g/100 g DW, respectively. These results are similar to those of previous studies (KABULOV and TASHBEKOV, 1979; EZE-

Table 1 - Fatty acid composition (%) of *P. oleracea* leaves.

Myristic acid (14:0)	5.04
Palmitic acid (16:0)	9.72
Stearic acid (18:0)	4.36
Oleic acid (18:1ω9)	8.83
Linoleic acid (18:2ω6)	14.01
Linolenic acid (18:3ω3)	56.33
Σ Fatty acid	98.29
Σ Saturated fatty acid	19.12
Σ Unsaturated fatty acid	79.17

KWE *et al.*, 1994; MOHAMED and HUSSEIN, 1994). It is well known that protein levels in *P. oleracea* accessions are comparable to or higher than those of other forage or vegetable food crops traditionally used as protein sources for humans and animals (MOHAMED and HUSSEIN, 1994).

The total lipid content in *P. oleracea* leaves was 5.83%. It has been reported that *P. oleracea* is the richest source of total lipids among wild plants (KESDEN and WILL, 1987). The total lipid content in leaves harvested under natural field conditions in this experiment were similar to the results reported by EZEKWE *et al.* (1994).

The results of the fatty acid analysis show that *P. oleracea* leaves contain six major fatty acids as reported in Table 1. Linolenic acid was the dominant fatty acid followed by linoleic, palmitic, oleic, myristic and stearic acids. GUIL-GUERRERO and RODRIGUEZ-GARCIA (1999), PALANISWAMY *et al.* (2001) and SIMOPOULOS (1991) reported that the main fatty acids in the that *P. oleracea* leaves are linolenic, linoleic, stearic, palmitic and oleic acids. The *P. oleracea* leaves were particularly rich in unsaturated fatty acids. The results of the study also revealed significant differences in the above-mentioned fatty acid concentrations. These results are consistent with those of OMARA-ALWALA *et al.* (1991), who reported significant differences in fatty acid concentrations and in the saturated/unsaturated ratios. They also reported the presence of eicosapentaenoic, docosapentaenoic and docosahexaenoic acids in *P. oleracea* leaves. These fatty acids were not detected in this study. Plant cultivars, sample material, sampling procedures, and/or analytical methods including sample preparation procedures and chromatographic conditions may affect the fatty acid profile.

The ascorbic acid (vitamin C) content in fresh *P. oleracea* leaves was 77.25 mg/100 g FW. In previous studies, the ascorbic acid content of *P. oleracea* was reported to be between 25-85 mg/100 g FW (SIMOPOULOS, 2004; LIM and QUAH, 2007). The ascorbic acid content in this study is within these limits. Wild plants are typically known to have higher vitamin C levels than cultivated ones (EATON and KONNER, 1985).

The results of free radical scavenging activity of *P. oleracea* leaves are reported in Table 2. The statistically significant reduction in the stable radical DPPH to yellow-colored diphenylpicrylhydrazine was obtained with an IC_{50}=54.33 μg/mL compared to 19.07, 18.65, 6.98 and 4.41 μg/mL for BHA, BHT, curcumin and ascorbic acid, respectively. This indicates that extracts of purslane are electron donors so they can react with free radicals, convert them into more stable products and terminate the radical chain reaction. This may be important in protecting cellular

Table 2 - Free radical scavenging capacities of the extracts measured in DPPH assay (n=6).

Extracts	IC_{50}(μg/mL)
P. oleracea leaves	54.33±1.26[a]
BHA	19.07±0.61[b]
BHT	18.65±0.49[b]
Curcumine	6.98±0.07[c]
Ascorbic acid	4.41±0.07[c]

*Values in the same line with different lower-case letters are significantly different at P<0.05.

Table 3 - Inhibition ratio of the linoleic acid oxidation by the extract (n=6).

Extracts	Inhibition ratio (%)
P. oleracea	91.15±2.02^{NS}
BHA	98.16±2.11
BHT	96.66±2.09
Curcumine	90.66±1.78
Ascorbic acid	93.87±1.69
NS: Not significant at $P<0.05$.	

DNA, lipids and proteins from free radical damage.

There were 17.88 μgGAE/mg DW equivalent of phenolic compounds in the extract. The antioxidative effectiveness in natural sources is mostly due to the phenolic content (HAYASE and KATO, 1984). The results for the total phenolic content in leaf extracts show that this plant is one of the richest phenolic sources. LIM and QUAH (2007) previously reported that total phenolic contents of ornamental *P. oleracea* plants grown in Malaysia were between 127-478 mg GAE/100 g FW. The variability could be due to the plants used, environmental factors and the collection period. Previous studies have indicated that the level of active compounds in plants increases as sunlight and temperature increase (BEN-HAMMOUDA *et al.*, 1995; DRAGLAND *et al.*, 2003). Phenolic compounds are antioxidants that contribute to the high antioxidant capacity observed in edible plants (SIMOPOULOS, 2004). Phenolics also play an important role against chronic diseases (NIJVELDT *et al.*, 2001).

In the β-carotene/linoleic acid assay, there were no statistically significant differences between extract, BHA, BHT, curcumin and ascorbic acid (Table 3). The inhibition ratio was 98.16% for BHA followed by BHT (96.66%), ascorbic acid (93.87%), *P. oleracea* extract (91.15%) and curcumin (90.66%). This result suggests that in the β-carotene/linoleic acid assay, oxidation of linoleic acid was effectively inhibited by the *P. oleracea* extract. *P. oleracea* leaves have a strong antioxidant capacity and can be important for the human diet and food safety when mixed with the other foods. In previous studies conducted on different cultivars of *P. oleracea*, the antioxidant activity was between 60-87% in β-carotene bleaching assay (LIM and QUAH, 2007). These results suggest that the consumption of *P. oleracea* could offer some dietary benefits since they contain antioxidant constituents that can protect against lipid peroxidation and scavenge free radicals. This report also suggested that *P. oleracea* could have beneficial chemopreventive effects in addition to providing potential new sources of natural antioxidants.

The mineral contents of *P. oleracea* leaves are shown in Table 4. In this study, potassium (K) was predominant (3,250 mg/100 g DW) followed by calcium (Ca) (328 mg/100 g DW) and phosphorus (P) (267 mg/100 g DW). In contrast to this MOHAMED and HUSSEIN (1994) reported that P was dominant in *P. oleracea* leaves. This could be due to the low P content in Erzurum plain soils which are rich in potassium. KESDEN and WILL (1987) reported a calcium con-

Table 4 - Mineral element content in *P. oleracea* leaves.

Mineral Elements (mg/100 g DW)								
P	K	Ca	Mg	Fe	Na	Mn	Zn	Cu
267	3,250	328	148	117	28	18	10	1

tent in *P. oleracea* plants of 79 mg per 100 g dry weight. The mineral composition of the plants depends, not only on the species or variety, but also on the growing conditions such as soil and geographical conditions.

The antibacterial activity of *P. oleracea* leaves against a number of bacteria is shown in Table 5. The most pronounced activity was shown by the methanolic extracts of *P. oleracea* against *Pseudomonas syringae* pv. *tomato, Bacillus subtilis, Vibrio cholerae* and *Yersinia pseudotuberculosis.* However, none of the water extracts showed any antibacterial activity against the microorganisms studied. The extracts did not show any antibacterial activity against *P. syringae pv. syringae, B. ceraus, Y. enterocolita, C. diphteriae, Y. frederiksenii, S. typhimurium, S. liquefaciens, P. corrugate, X. compestris compestris, A. tumafaciens* and *Pseudomonas*.

MIC values of the methanol extract and standard drug (maxipime) ranged from 125 to 250 μg/mL and from 7.81 to 500 μg/mL, respectively (Table 5). *Xanthomonas compestris compestris* was the most sensitive to the plant extract, with a MIC value that was lower than the standard drug. *Pseudomonas syringae* pv. *syringae, Pseudomonas syringae* pv. *tomato* and *Pseudomonas aeruginosa* were also sensitive with MIC values that were similar to those of the standard antibiotic (Maxipime).

Prior to this study there have been no reports about the antibacterial properties and use of *P. oleracea* against a number of bacteria. The results obtained in the present study are in agreement to a certain degree with the medicinal uses of *P. oleracea.* This is the first report of data that *P. oleracea* extracts have potential antibacterial activities against a number of bacteria.

In conclusion, extracts of wild *P. olerecea* plants have high total phenolic, ascorbic acid, total lipid, protein and fatty acid contents and display antioxidant activities. Antibacterial activity was also observed. Therefore wild *P. oleracea* plants may provide a new source of antioxidant and antibacterial agents as

Table 5 - Antibacterial activity of *P. oleracea* leaf extracts against a number of bacteria.

Bacterial species	Inhibition zone in diameter (mm) of *P. oleracea* extracts (10 μg/disc)	Water extract	Negative control MeOH	Positive control (mm) Net	Sfc	Ofx	MIC values (μg/mL) Extract	Standard drug Maxipime
Pseudomonas syringae pv. *syringae*	-	-	-	16	-	16	250	250
Pseudomonas syringae pv. *tomato*	7	-	-	20	-	-	250	250
Bacillus subtilis	7	-	-	30	40	26	250	7.81
Bacillus cereus	-	-	-	19	19	21	250	31.25
Yersinia enterocolita	-	-	-	-	19	-	250	31.25
Vibrio cholerae	7	-	-	16	39	21	125	62.50
Coryneum diphteriae	-	-	-	-	-	-	250	-
Yersinia frederiksenii	-	-	-	12	19	18	250	31.25
Yersinia pseudotuberculosis	7	-	-	17	12	16	250	31.25
Salmonella typhimurium	-	-	-	-	9	-	250	-
Serratia liquefaciens	-	-	-	14	-	10	250	62.50
Pseudomonas corrugata	-	-	-	13	10	23	250	-
Xanthomonas compestris compestris	-	-	-	9	15	15	250	500
Agrobaceterium tumefaciens	-	-	-	16	25	23	250	-
Pseudomonas aeruginosa	-	-	-	14	12	17	250	250

well as genes that can be used for food and medicinal purposes. Sampling date and phenotype factors should be considered in future efforts to improve and domesticate *P. oleracea* as a major agricultural crop.

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PRELIMINARY SURVEY ON NUTRITIONAL AND COMMERCIAL FEATURES OF PEACHES FROM EMILIA ROMAGNA: ANTIOXIDANT PROPERTIES AS A POSSIBLE NOVEL QUALITY PARAMETER

INDAGINE PRELIMINARE SULLE CARATTERISTICHE NUTRIZIONALI E COMMERCIALI DELLE PESCHE PRODOTTE IN EMILIA ROMAGNA: LE PROPRIETÀ ANTIOSSIDANTI COME POSSIBILE INNOVATIVO PARAMETRO DI QUALITÀ

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ABSTRACT

The aim of this study was to screen and compare 50 different genotypes of peaches by measuring their total antioxidant contents (TAC) and total polyphenolic contents (TPH) immediate-

RIASSUNTO

Lo scopo dello studio era lo screening ed il confronto di 50 diversi genotipi di pesche misurando la loro capacità antiossidante totale (TAC) ed il contenuto di polifenoli (TPH) dopo la raccolta e

- Key words: healthy diets, post-harvest, sugar/acidity ratio, TEAC, total antioxidant capacity, total phenol content -

ly after harvesting and upon cold-storage; the relation between TEAC vs sugar/acidity ratio was also determined. Most of the cultivars (30) displayed very similar TEAC values, between 0.36 mM Trolox /g FW and 0.83 mM Trolox /g FW. Only 5 cultivars had lower values and the remaining 15 had higher TAC. Some of the latter were characterized by very high TEAC values and Emeraude, Laura, Rosa del West, Maria Delizia, Flavor Crest and Iris Rosso displayed TAC values that were several times higher than the value of most of the cultivars analysed. The TPH values were also distributed in a limited range; in fact 42 out of 50 cultivars displayed TPH values between 0.5 and 1.5 mg GA/g fruit. The nutritional quality of post-harvest peaches suggess that short storage (7 days from harvesting) may maintain or even slightly increase TAC, while longer storage (30 days from harvesting) may reduce baseline values. TAC and TPH values in peaches indicate a variability in nutritional value among peach cultivars. Proper evaluation of these characteristics could lead to the identification of a number of genotypes that would improve the nutritional features of fruit intake. These characteristics could be valuable to the consumer, when assessing healthy (antioxidant-enriched) diets.

la conservazione. La maggior parte (30) presentava valori molto simili di TEAC (tra 0,36 e 0,83 mM Trolox/g FW). Solo 5 cultivars avevano valori inferiori a questo intervallo e 15 superiori. Emeraude, Laura, Rosa del West, Maria Delizia, Flavor Crest and Iris Rosso presentavano valori particolarmente elevati. I valori di TPH erano in un intervallo tra 0,5 ed 1,5 mg GA/g frutta in 42 cultivars. La conservazione post-raccolta può influire su questi valori. L'identificazione di genotipi in grado di esprimere caratteristiche nutrizionali migliorate può essere utile per il consumatore quando è necessario predisporre diete salutari ricche in antiossidanti.

INTRODUCTION

The important role of diet in promoting or preventing disease has long been recognised. Global epidemiological studies correlate the prevalence of certain pathologies to dietary habits and confirm an inverse relationship between the consumption of fruit and the incidence of many chronic illnesses. Diet plays a role in protecting against cardiovascular (JOHNSEN *et al.*, 2003; HEINONEN *et al.*, 1998), degenerative (DE RUVO *et al.*, 2000) and proliferative diseases (SMITH-WARNER *et al.*, 2003; CHU *et al.*, 2002). Although the outcome of epidemiological studies is not always unequivocal, there is convincing evidence that the considerable health benefits of fruit are due to their specific chemical composition,

particularly to compounds of nutritional relevance.

Consumers are now aware that the consumption of fruit that is rich in health-promoting compounds is an appropriate strategy for enjoying its benefits. Thus, the nutritional quality of fruit is becoming an attribute as important as that of the organoleptic-sensorial quality, even though it is an extremely complex parameter.

Peaches have been part of the human diet for centuries and are a potentially important contributor to the intake of fresh fruit for populations in countries where there is a limited availability of fruit and vegetables, e.g. in the northern latitudes. Peaches are among the most commonly consumed fruit, particularly during the summer season.

As other fruit, they are an important dietary source of fiber and micronutrients such as essential minerals and vitamins, and play a relevant role in human health by protecting essential biomolecules from damage induced by free radicals and reactive oxygen species, and in regenerating the active form of other molecules such as vitamin E (CARR and FREI, 1999). Peaches are characterised by a moderate concentration of phenolic compounds which are usually regarded as secondary plant metabolites, which are not essential to the survival of the plant. These compounds may, however, strongly influence fruit quality, by contributing to their sensorial-organoleptic attributes (flavour, aroma, color) and to their nutritional quality (SCALZO *et al.*, 2005; DEIGHTON *et al.*, 2000). Phenolic compounds are not like vitamins, in that they are not required for normal functioning of the human body and their absence does not result in a condition of deficiency. However, they do have marked bioactivities in mammalian cells which could have an impact on health and disease. Some potential health benefits of peaches involve their effect on oxidative damage, detoxification enzymes, gene expression modulation and anti-inflammatory, anti-bacterial and anti-viral responses.

It seems that the total antioxidant capacity (TAC) characteristic greatly depends on the genetic base (species-variety) and is influenced by cultivation method and techniques, as well as by storage technique and duration. Therefore it could be very important to increase peach production considering its novel qualitative parameters and/or nutritional properties such as TAC and phenol contents. A rational procedure for assessing the nutritional quality of fruit should start with phytochemical and antioxidant profiling and only subsequently pass to bioavailability and bioefficacy studies. The chemical and antioxidant profile of peaches, and the influence of genetic differences among cultivars will lead to a better understanding of the role of these substances in the physiology and organoleptic-sensorial quality of the fruit.

The aim of the present work was to screen and compare 50 different genotypes of peaches by measuring their TAC contents and total polyphenolic contents (TPH), as well as to determine the relationship between TAC vs sugar/acidity ratio.

METHODS AND MATERIALS

Chemicals

2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt (ABTS), Trolox, Folin-Ciocalteu phenol reagent and anhydrous sodium carbonate were purchased from Fluka Chemie GmbH (Buchs, Switzerland); 3,4,5-trihydroxybenzoic acid (gallic acid; GA), potassium persulfate, and all solvents (high-performance liquid chromatographic grade) were obtained from Sigma Aldrich s.r.l. (Milan, Italy).

Peach samples

Fifty different peach cultivars were compared; all fruit analyzed was harvested from plants grafted on a GF677 rootstock, 37 of the 50 peach cultivars used were of "Protected Geographical Indication".

Peach or nectarine cultivars were characterized by white or yellow pulp as follows:

- Douceur, Iris Rosso, Alirosada, Maria Delizia, Rosa Del West (white flesh);
- Crimson Lady, Diamond Princess, Elegant Lady, Fayette, Fayette P, Flavour Crest, Grenat, Kaweah, Maria Bianca, Maria Marta, May Crest, Nadia, Red Moon, Redheven, Rich Lady, Royal Gem, Royal Glory, Springbelle, Springcrest, Suncrest, Synphonie. (yellow flesh);
- Caldesi 2000, Emeraude, Jade, Silver Giant, Zephyr. (white nectarine);
- Ali Top, Ambra, Big Bang, Big Top, Diamond Ray, Guerriera, Laura, Maria Aurelia, Maria Carla, Max 7, Nectaross, Orion, Springbright, Stark Red Gold, Summergrand, Supercrimson, Sweet Lady, Sweet Red, Venus (yellow nectarine).

Fifty peaches were picked per cultivar; 25 peaches were used for the evaluation of qualitative/commercial parameters: fruit weight, consistency, peel colour, soluble solids (%SS) using a handheld refractometer and titratable acidity (TA) (mEq NaOH/100 g fresh fruit) using 10 mL peach juice diluted with distilled water (1:2) that was titrated to pH 8.2 against 0.1N NaOH.

The remaining 25 peaches were immediately frozen at -20°C after picking in order to stop any possible enzyme degrading action.

To test the stability of the nutritional quality parameters during the postharvest treatments, fruit of two cultivars (Fayette and Stark Red Gold) were also analysed after a standard cold-storage treatment used before large-scale retail trade. The fruit in cold storage was kept for 7, 15 or 30 days at room temperature between -0.5°/+0.5°C with 90-95% relative humidity. The fruit temperature was always between 2° and 3°C.

ABTS assay

Total antioxidant capacity of whole fruit extracts was measured as Trolox Equivalent Antioxidant Capacity (TEAC) using 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt (ABTS), a radical cation. The contributions of both the hydrophilic and lipophilic fractions were determined.

TAC was evaluated according to the ABTS modified assay (BOMPADRE *et al.*, 1999; RE *et al.*, 1999). ABTS, a chromogen and a colorless substance, is changed into its colored monocationic radical form ($ABTS^{\cdot+}$) by an oxidative agent. The absorption peak of $ABTS^{\cdot+}$ is at 734 nm. Addition of antioxidants reduces $ABTS^{\cdot+}$ to its colorless form. The extent of decolorization as percentage of inhibition of $ABTS^{\cdot+}$ is determined as a function of concentration and calculated relative to the reactivity of Trolox, a watersoluble analog of vitamin E. Antioxidant activity is expressed as micromoles of Trolox equivalents per gram of fresh pulp weight. Following PELLEGRINI *et al.* (1999), the ABTS radicalized solution (ABTS·+) was prepared by reacting ABTS (7 mmol/L) with $K_2S_2O_8$ (140 mmol/L) to obtain a molar ratio of 1:0.35. The $ABTS^{\cdot+}$ working solution was prepared by dissolving $ABTS^{\cdot+}$ radicalized solution in ethanol (1:100). A dose-response curve was derived for Trolox (0, 0.25, 0.5, 1.0, and 1.5 mmol/L) diluted in 1 mL of ABTS·+ working solution.

Sample preparation and procedure

To assess the antioxidant power of the fruit, two types of extraction, hydrophilic and lipophilic, were performed. Hydrophilic extraction was performed with different amounts (0.4

to 1 g) of homogenized fruit that were diluted with methanol and water (80% v/v) to a final weight to volume ratio of 1:10. The pH of each sample was adjusted with 1 mol/L of NaOH until it reached 5.0±0.2, and then the samples were centrifuged at 3500g for 10 min. The supernatant and pellet of each sample were recovered. Lipophilic extraction was done on a pellet by adding acetone (weight to volume ratio of 1:2 or 1:5, according to the type of fruit) and then centrifuging for 15 min at 3,500 g. Each supernatant and pellet were recovered, and the antioxidant capacity of each was measured separately by recording absorbance at 734 nm on a spectrophotometer (Kontron Uvikon 941 Plus, Milano, Italy).

The antioxidant capacity of the fruit is expressed as Trolox equivalent antioxidant capacity (TEAC) (SCALZO *et al.*, 2005) by using the calibration curve plotted against different amounts of Trolox. TEAC values were calculated and are expressed using Trolox equivalents (TE) per gram of fresh weight (FW).

Data are expressed as antioxidant capacity induced by hydrophilic and lipophilic components, and total antioxidant capacity as the sum of the two phases.

Total phenolic content (TPH) of fruit

Phenolic content was determined by the Folin-Ciocalteu method (SLINKARD and SINGLETON, 1997) by using GA as a standard for the calibration curve. Results were calculated and are expressed as milligrams of GA equivalent (GAE) per gram of fresh weight. Samples (fruit juice) or standards (0, 0.6, 1.2, 1.8, 2.4, and 3 mmol/L of GA) diluted 1:10 were added to the Folin-Ciocalteu reagent (diluted 1:10) to obtain a 1:5 ratio (v/v) after 1 min and before adding sodium carbonate solution (700 mmol/L) and incubating for 8 min (2:3 v/v). Results were read at 760 nm after 2 h.

RESULTS AND DISCUSSION

Nutritional parameters

The lipophilic and hydrophilic extracts of each of the 50 peach genotypes were subjected to TEAC and TPH analysis. For each sample the analysis was repeated 8 times i.e. (a total of 1200 analyses). Most of the cultivars (30) displayed very similar TEAC values, between 0.36 mM Trolox /g FW (Suncrest) and 0.83 mM Trolox /g FW (Rich Lady). Five cultivars had lower values and the remaining 15 had higher TAC values. Some of the latter were characterized by very high TAC values such as Emeraude (1.4394 mM Trolox/g FW), Laura (1.5239 mM Trolox/g FW), Rosa del West (1.7499 mM Trolox/g FW), Maria Delizia (1.9724 mM Trolox/g FW), Flavor Crest (2.0283 mM Trolox/g FW), and Iris Rosso (2.3493 mM Trolox/g FW). In fact, these cultivars displayed TEAC values that were several times greater than those recorded for the majority of the cultivars analyzed (Fig. 1).

TAC can be strongly affected by the relative antioxidant composition of each cultivar. This is reflected in the corresponding contribution to TAC made by the wide hydrophilic and lipophylic pool (Fig. 2). As can be seen that the two contributions vary greatly. Each cultivar is characterized by the contribution of different percentages of the two components to TAC; this is valuable information if production, characterized by specific antioxidant features, is required.

For example, regardless of the final TEAC, some cultivars were extremely rich in lipophilic antioxidants; in the case of Big Top and Royal Gem the lipophilic contribution was even higher than the hydrophilic one. In other cultivars the lipophilic contribution accounted for 30-40% of TEAC (Caldesi 2000, Springbright, Royal Glory, Maria Carla, Rich Lady, Red Moon, Guerriera), while in most cases it accounted for less than

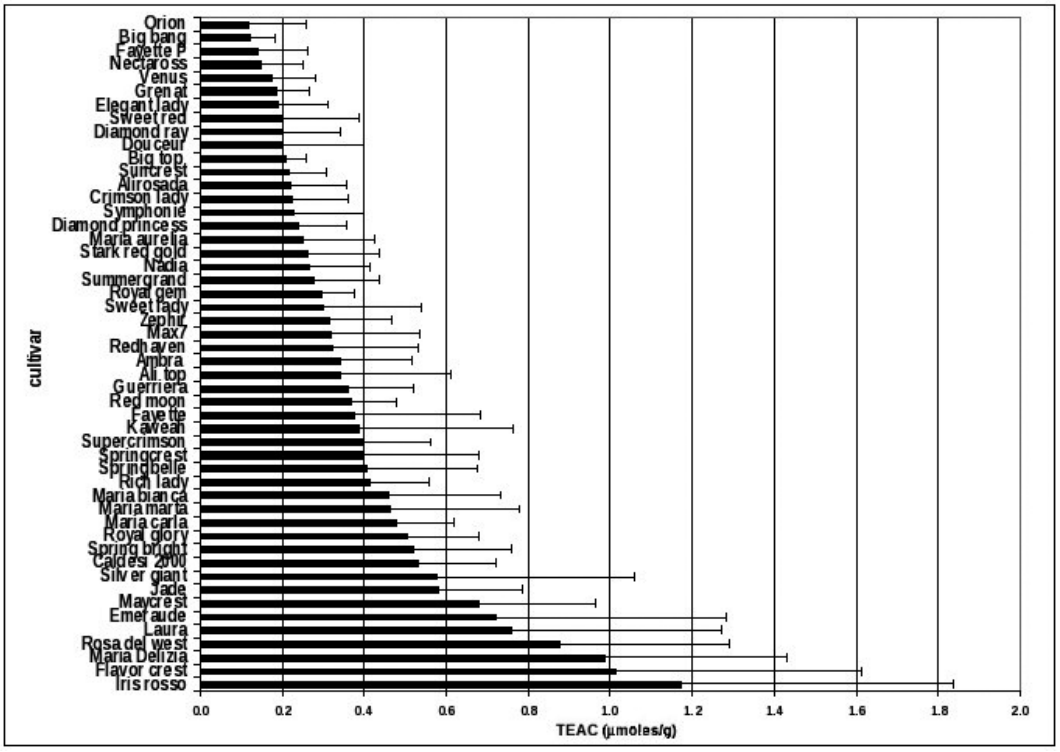

Fig. 1 - Total TEAC values expressed in Trolox E (µmoles/g). Each value is the average of 8 replications ± standard deviation.

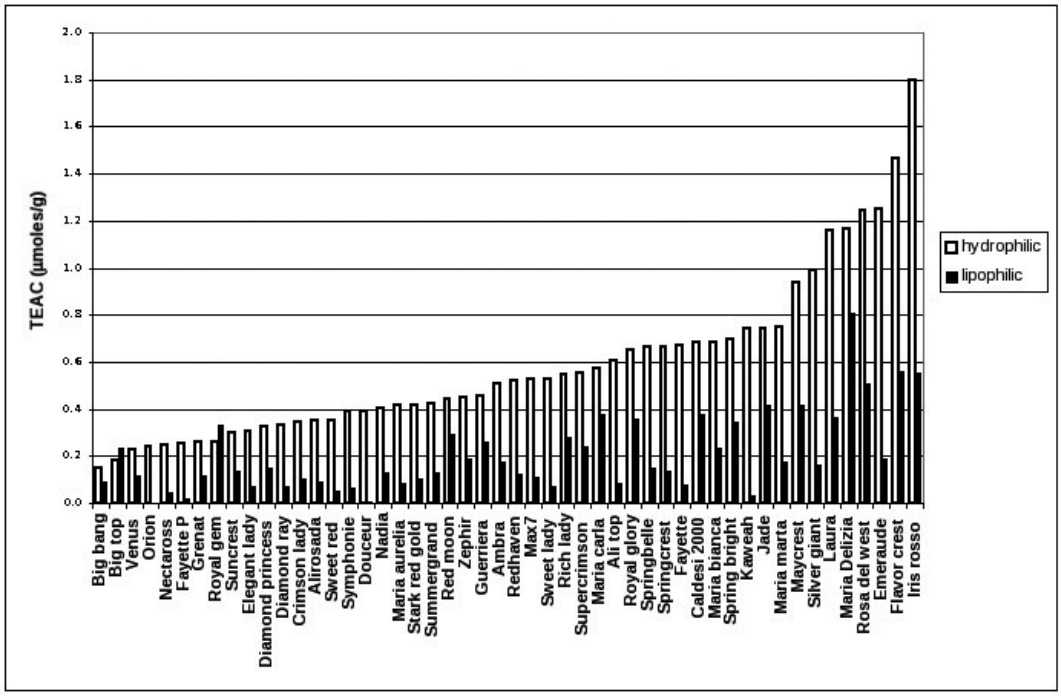

Fig. 2 - Hydrophilic and lipophilic TEAC values expressed in Trolox E (µmoles/g).

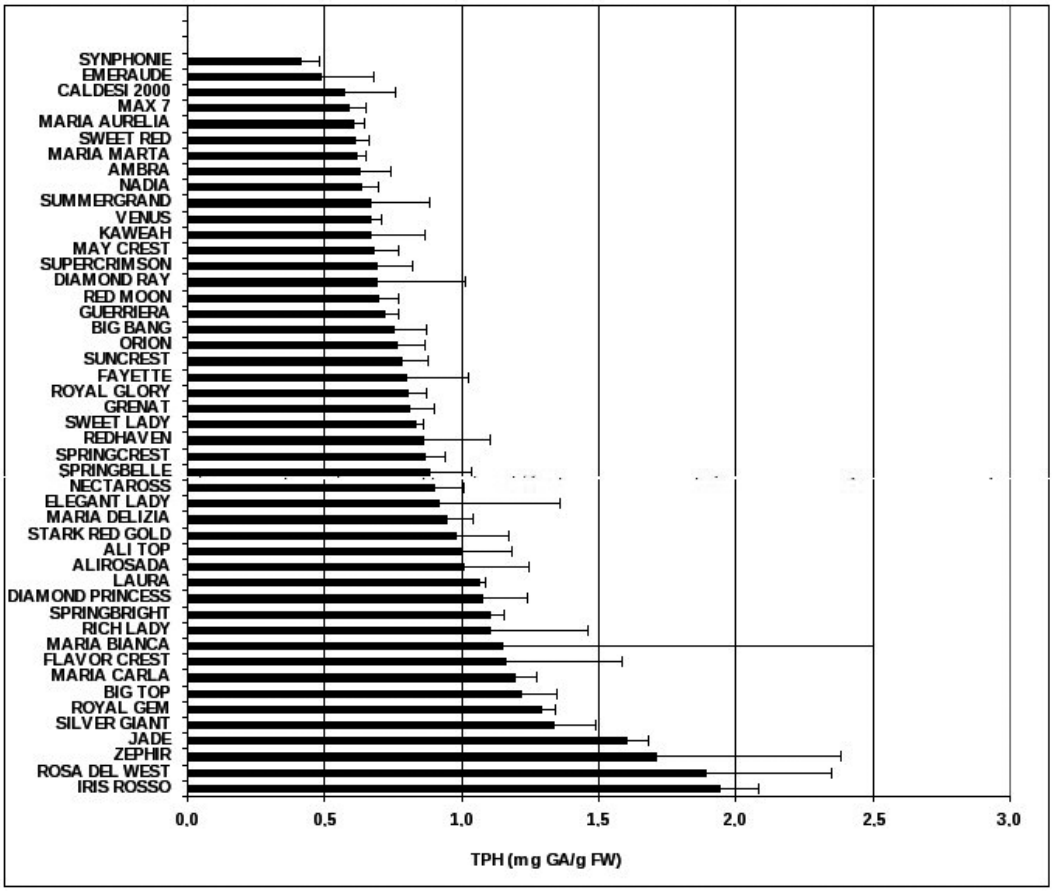

Fig. 3 - Total TPH values expressed in mg GA/g fruit. Each value is the average of 4 replications ± standard deviation.

10% of TEAC. Finally, the lipophilic contribution was negligible in a few cultivars (Orion, Fayette P, Fayette, Kaweah).

The TPH values were also distributed in a limited range of concentration; 42 of the 50 cultivars displayed TPH values between 0.5 and 1.5 mg GA/g fruit. Moreover, only Fayette P and Crimson Lady had TPH concentrations that were twice the average value (Fig. 3). The TPH values fell within the range of concentrations already indicated in the literature (GIORGI *et al.*, 2005; BATTINO and MEZZETTI, 2006).

Nutritional quality in post-harvest peaches

Peaches were kept in cold storage for different lengths of time: Fayette was analyzed after 7 and 15 days of storage, while Stark Red Gold was analyzed after 15 and 30 days of storage. The results suggest that after a short storage time (7 days after harvest) TAC may remain stable or even increase slightly, while after longer storage time (30 days after harvest) the TAC values decreased (Fig. 4a and 4b).

Finally, important commercial parameters (expressed as sugar/acidity ratio) were considered in view of the corresponding TAC for each cultivar. No correlation was found and in other kinds of fruit (BATTINO and MEZZETTI, 2006); the varieties characterized by high commercial features are not always efficient with respect to antioxidant capacity.

In fact, higher SS/TA ratios which represent a better acceptance by the consumers are rarely associated with high

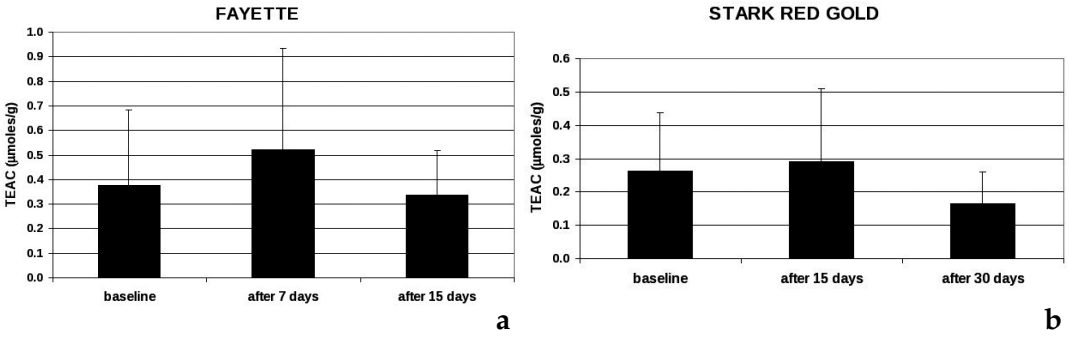

Fig. 4a-b - The effect of cold-storage on TEAC values in Fayette and Stark Red Gold.

TEAC. It is interesting to note that the upper right quadrant in Fig. 5, representing excellence for a fruit, is completely empty; this could be a target for breeders in the near future.

It has been demonstrated that rootstocks may significantly affect TEAC of both peaches and apricots (BATTINO and MEZZETTI, 2006; GIORGI *et al.*, 2005). For this reason all of the cultivars in this study had the same rootstock (GF 677) in order to avoid any effect. However, in order to modulate TEAC, in future studies it could be useful to use different rootstocks.

The intake of antioxidants through fruit consumption may be of great importance in many diseases, pathological status or dietary deficiencies since their presence directly affects human health and well-being (BATTINO and MEZZETTI, 2006; GREABU *et al.*, 2007; BATTINO *et al.*, 1999). Therefore the availability of high quality fruit (antioxidant-enriched) will be useful in the planning of healthy diets especially when patients do not eat a sufficient amount of fruit, when an attractive, tasty alternative, is needed, peaches are usually used.

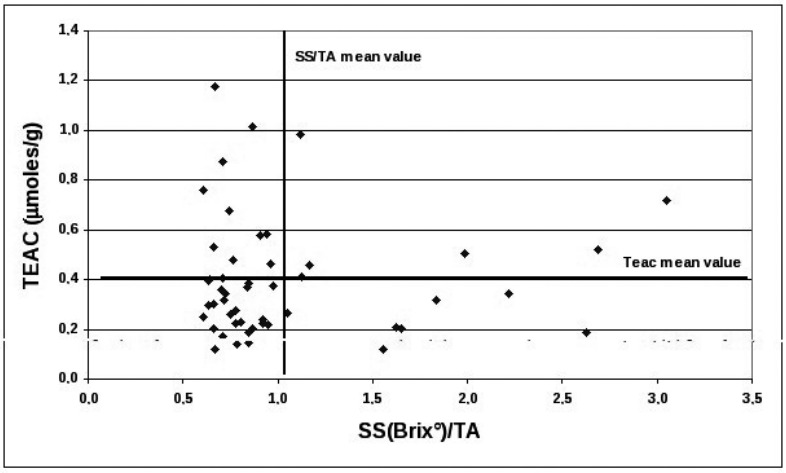

Fig. 5 - Sugar (°Brix)/titratable acidity ratio *vs* TEAC expressed in Trolox E (µmoles/g).

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EVALUATION OF SOME HEAT-TREATMENT INDICES IN UHT MILK MARKETED IN ITALY

VALUTAZIONE DI ALCUNI INDICI DI TRATTAMENTO TERMICO NEL LATTE UHT COMMERCIALIZZATO IN ITALIA

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ABSTRACT

A survey on heat damage of both direct and indirect UHT milk marketed in Italy was carried out. Furosine, lactulose, galactosyl-β-pyranone and lysinoalanine were determined in order to quantify the severity of heat processing in 92 UHT milk samples. Twenty-seven samples out of ninety-two were imported from other countries and showed value ranges for these parameters similar to those manufactured in Italy. Anomalous levels for at least one of the four parameters were revealed

RIASSUNTO

In questo lavoro sono riportati i risultati di un'indagine sul danno termico del latte sterilizzato UHT, sia di tipo diretto che indiretto, commercializzato in Italia. Su 92 campioni di latte sono stati determinati i valori di furosina, lattulosio, galattosil-β-piranone e lisinoalanina. Ventisette dei 92 campioni erano prodotti all'estero e hanno presentato valori di questi indici simili a quelli riscontrati per il latte prodotto in Italia. Almeno uno dei quattro indici ha presentato valori anomali nel 40%

- Key words: furosine, galactosyl-β-pyranone, lactulose, lysinoalanine, UHT milk -

in about 40% of the samples, indicating the use of operating conditions that promote relevant heat damage. While the adoption of regulations to guarantee the quality of UHT milk does not seem to be a priority of EU policy, fixing the threshold values for one or more of these indices is recommended as a minimal quality requirement for UHT milk in order to prevent improper technological practices.

dei campioni prodotti in Italia, suggerendo l'avvenuto utilizzo da parte dei produttori di condizioni di lavorazione tali da promuovere un eccessivo danno termico. L'adozione di un valore soglia massimo per uno o più di questi parametri è il criterio proposto per monitorare i requisiti minimi di qualità del latte sterile UHT.

INTRODUCTION

About 55% of the drinking milk sold in Italy during 2007 was UHT milk (ASSOLATTE, 2008), probably because of its prolonged shelf life. The quality of UHT milk mainly depends on the level of heat damage which is strictly correlated to the severity of the heat treatment applied during manufacturing. In fact, heat treatment of milk leads to chemical and physical changes, which in turn, alter both the sensory properties and nutritional value, and may yield unnatural molecules whose properties are not yet fully understood (FRIEDMAN, 1999).

Legal specifications intended to preserve the quality of UHT milk are very weak. The latest Regulations (EC) No. 852/2004 and No. 853/2004, became effective in January 2006 and were amended by Regulation (EC) No. 2074/2005. These regulations lay down hygiene rules for food of animal origin. As far as milk and dairy products are concerned, prescriptions are given for the hygiene and safety of finished products in order to protect human health. No definitions for "heat treatment" or "heat-treated drinking milk" are given. Furthermore, since no upper limits were set for the time/temperature requirements for the different types of drinking milk, it is not possible to control the end products placed on the market. The products cannot be objectively evaluated and no guarantee can be given to the consumer regarding their quality in terms of heat damage. More regulatory attention has been given to the fat content (EC REGULATION, 2007).

While ITALIAN LAW No. 169 (1989) established quality parameters for three different types of pasteurized milk produced in Italy, guaranteeing the minimum heat damage in these products, no equivalent parameters were established for UHT milk as it was of minor interest at that time. This situation could allow manufacturers of UHT milk to increase time/temperature combinations of the heat treatment in order to prolong the shelf life of the finished product or to attain sterility of raw milk of poor microbiological quality.

CATTANEO *et al.* (2008) recently evaluated heat damage in UHT milk and showed evidence of unjustified milk overheating. Data obtained for UHT milk samples, processed under controlled conditions at different industrial plants, suggested the suitability of adopting heat-damage parameters for the authentication and quality assessment of commercial UHT milk.

The extent of heat damage of milk can be measured by means of suitable chemical indicators arising from heat-induced

reactions involving lactose and protein. Furosine (FUR), a marker of the early stage of the Maillard reaction, and lactulose (LCT), deriving from epimerization of lactose, are well known markers for heat damage in sterilized milk. Several researchers have proposed using a combination of these indices to obtain a better characterization of this type of milk (CORZO *et al.*, 1994; PELLEGRINO *et al.*, 1995).

More recently, galactosyl-β-pyranone (GAP), a molecule arising from the degradation of lactulosyl-lysine (Amadori compound) *via* the intermediate 1-deoxyosone (KRAMHÖLLER *et al.*, 1993; PISCHETSRIEDER *et al.*, 1999), has been proposed as a reliable index for evaluating the extent of advanced Maillard reaction in milk, and for distinguishing between direct and indirect UHT milk (RESMINI *et al.*, 2003).

Among unnatural amino acids formed by heat- or alkali-treated proteins, lysinoalanine (LAL) proved to be the most sensitive marker for both heat load and storage conditions of UHT milk (CATTANEO *et al.*, 2008). Furthermore, high levels of LAL in foodstuffs are considered detrimental from a nutritional point of view (FRIEDMAN, 1999).

In the present paper we report the measurements of the FUR, LCT, GAP and LAL levels in both direct and indirect UHT milk samples that were either manufactured in Italy or imported, and collected on the domestic market. The values found in the present survey are compared with those reported in the literature, particularly with those of ANDREINI *et al.* (1990) which refer to the quality of UHT milk marketed in Italy about 15 years ago.

MATERIALS AND METHODS

Ninety-two samples (65 samples manufactured in Italy and 27 imported from EU countries) of semi-skimmed (1.5-1.8% fat) UHT milk were collected on the Italian market and analyzed within 30 days of production. While the type of sterilization process (either direct or indirect) was confirmed by the manufacturers for the Italian milk samples, this was not possible for the imported samples.

The FUR level was determined by IP-RP HPLC according to ISO 18329-IDF 193 International Standard. Lactulose was determined by HPLC after removal of fat and protein (IDF Standard 147B). The GAP content was measured by the direct HPLC method of PELLEGRINO and CATTANEO (2001). LAL was determined by RP-HPLC of the FMOC-derivative with fluorescence detection (PELLEGRINO *et al.*, 1996). The analyses were carried out in duplicate on all milk samples and the mean values are reported.

RESULTS AND DISCUSSION

In contrast to what was reported by ANDREINI *et al.* (1990), at present, direct UHT milk largely prevails over indirect UHT milk on the Italian market. Although this survey does not cover the whole domestic market of UHT sterilized milk, the analyzed samples represent about 60% of the market (PELLEGRINO, 2007).

The distribution of FUR, LCT, GAP and LAL values found in a total of 92 samples of UHT milk are reported in Figs. 1-4. For direct UHT milk, the FUR levels (Fig. 1) ranged from 30 to 179 mg/100 g of protein (mean value: 94 mg/100 g of protein) and those of LCT (Fig. 2) from 109 to 726 mg/L (mean value: 298 mg/L). No comparison can be made with the values of FUR reported by ANDREINI *et al.* (1990), because a different analytical method was used, while the values of LCT ranged from 41 to 566 mg/L, with a mean value of 177 mg.

A range from 100 to 255 mg/100 g protein for FUR levels (mean value: 190

mg/100 g protein) and a range from 361 to 828 mg/L for LCT levels (mean value: 606 mg/L) were found in indirect UHT milk samples. In 1990 (ANDREINI *et al.*, 1990) the LCT levels of Italian indirect UHT milk ranged from 150 to 900 mg/L (with one sample reaching 1358 mg), with a mean value of 456 mg.

Comparing the LCT levels of UHT milk (Fig. 2) from this study with those of 1990 (ANDREINI *et al.*, 1990), it is evident that the average values are now much higher for both the direct and indirect types. In fact, referring to the indirect type, no samples were found that had less than 100 mg/L, while this val-

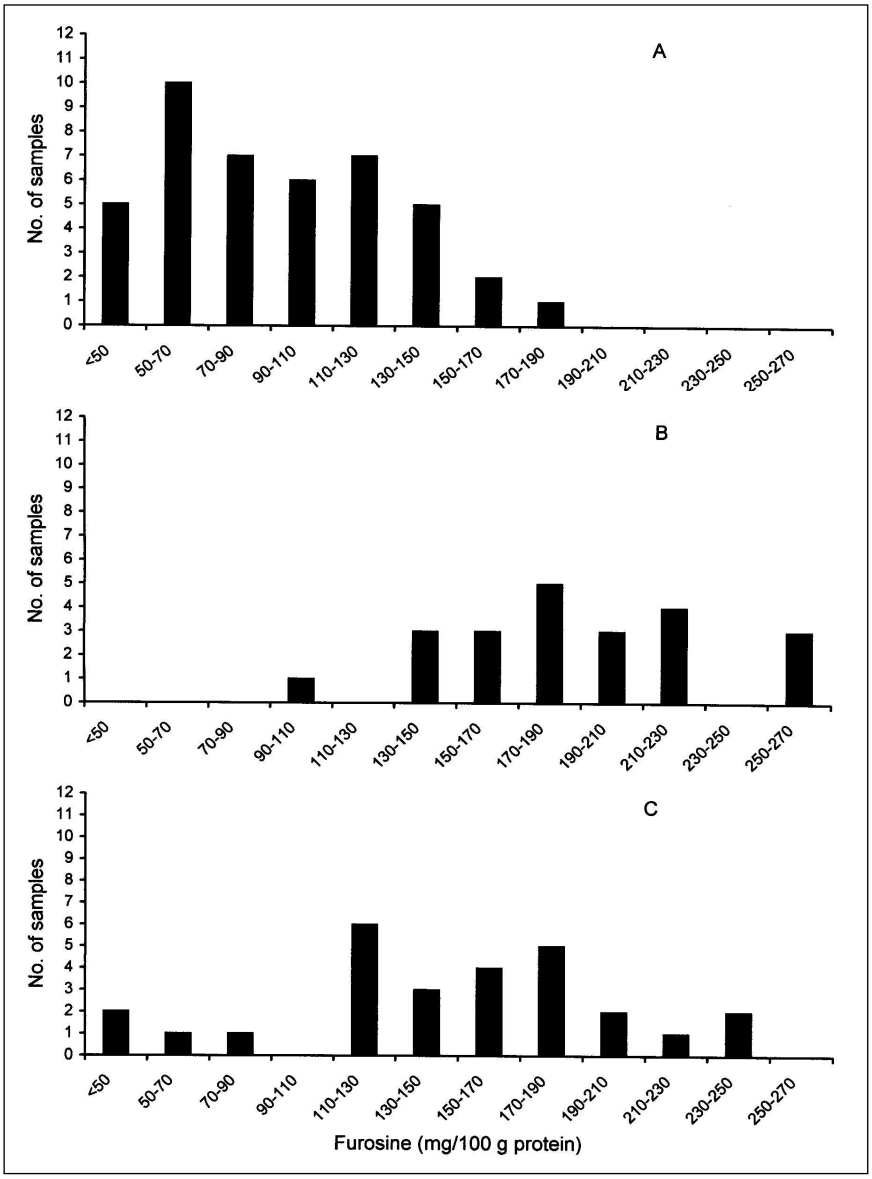

Fig. 1 - Levels of furosine in commercial UHT milk samples. Directly (A, n=43) or indirectly (B, n=22) heated and manufactured in Italy, and imported (C, n=27).

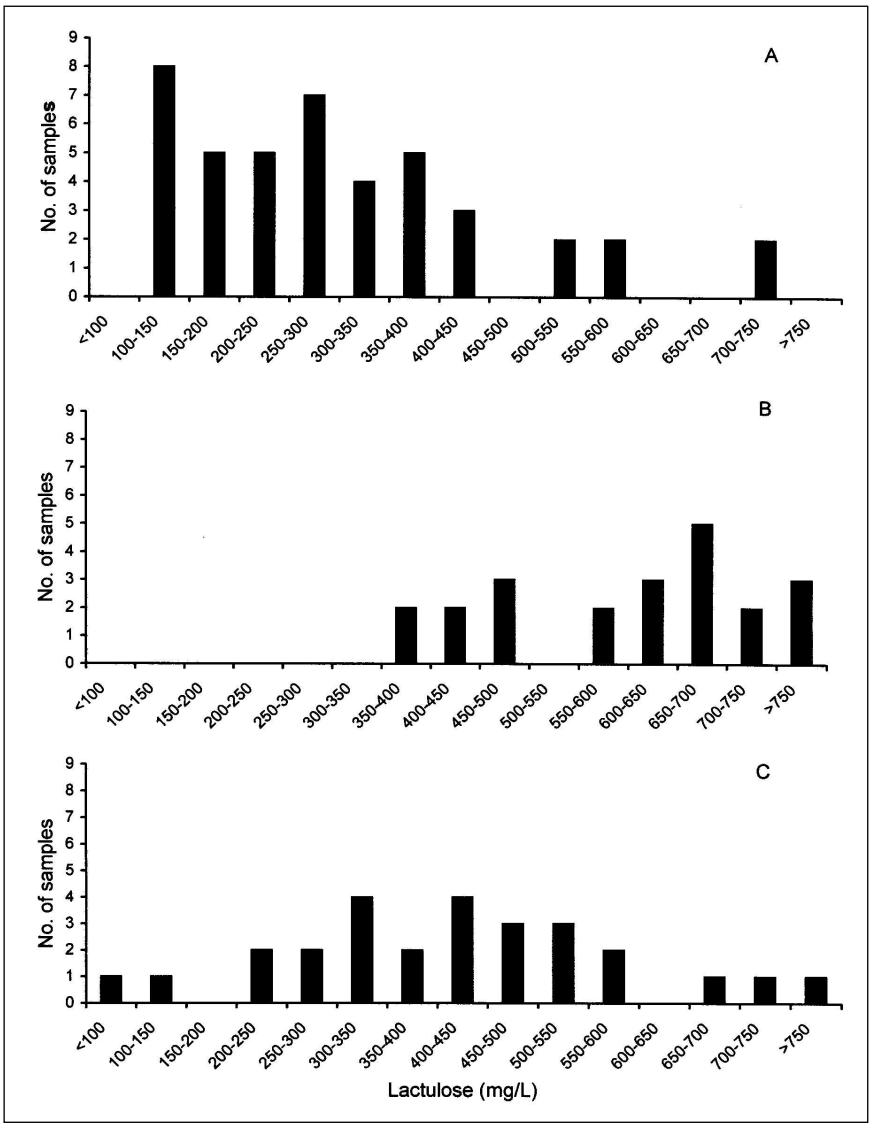

Fig. 2 - Levels of lactulose in commercial UHT milk samples. Directly (A, n=43) or indirectly (B, n=22) heated and manufactured in Italy, and imported (C, n=27).

ue accounted for 15% of the samples in the past. Furthermore, about 40% of the present samples had a LCT content greater than 300 mg/L, while only 10% of the samples had this value in the past. The comparison is consistent because the same analytical methods were used. With respect to indirect UHT milk, about 60% of the samples had a LCT content greater than 600 mg/L, while in the past, less than 20% of the samples had such a value. It should be noted that 600 mg/L was the upper limit for the LCT content in UHT milk proposed by the FIL-IDF and agreed upon by the EUROPEAN COMMISSION DAIRY CHEMIST'S GROUP (1993). Two samples of direct UHT milk largely exceeded this limit.

Therefore, based on the LCT levels, the

present survey shows that there is a substantial increase of heat damage in UHT milk produced in Italy. As expected, the FUR and LCT levels are lower in direct UHT milk samples than in the indirect ones, on average, because of the different heating system involved in manufacturing. The high dispersion of the data obtained for each type of milk is partly due to the heterogeneity of processing parameters (mainly nominal time/temperature conditions and the rate of milk recirculated in the plant) adopted by manufacturers, as well as to the wide range of sterilizing procedures at the different UHT plants (BURTON, 1988).

The FUR and LCT levels found in samples of UHT drinking milk imported from other countries (Germany, Austria, France, Slovenia and Czech Republic), not able to be distinguished as direct and indirect ones, showed distributions approximately covering the respective ranges found for the Italian samples. However, a few samples had very low levels of heat damage and no samples had the highest levels. Only 3 samples out of 27 (11%) showed a LCT content higher than 600 mg/L. These data prove that UHT milk imported into Italy is generally of good quality as far as the heat damage is concerned. This is because most of the countries of origin traditionally produce good quality UHT milk. Figures available for other countries show rather different situations although in all cases reference analytical methods were used. MORTIER *et al.* (2000) found FUR levels ranging from 60 to 200 mg/100 g protein and LCT levels from 100 to 820 mg/L for direct UHT milk marketed in Belgium, although the number of samples was not specified. In indirect UHT milk, the ranges were approximately 60-290 mg/100 g protein and 120-800 mg/L for FUR and LCT levels, respectively. A survey conducted in Spain in 1998 (RODRIGUEZ, 2007) on 348 samples of UHT milk (no distinction was made between direct and indirect heating systems) showed FUR levels ranging from 30 to 300 mg/100 g protein and LCT levels from 100 to 900 mg/L. In some cases FUR exceeded 300 mg/100 g protein and LCT exceeded 1,000 mg/L.

In a recent study (CATTANEO *et al.*, 2008) UHT milk samples were analyzed that had been processed at industrial plants under thermal conditions that intentionally caused severe heat damage to the finished products. It was found that the proposed upper limit of 600 mg/L for LCT was exceeded in direct UHT milk only when the process was nominally performed at 150°C for 9 s or longer. Under these conditions, the corresponding FUR levels were higher than 150 mg/100 g protein. Because of the different heating system, these levels were more easily exceeded in the case of indirect processing, e.g. when milk was heated at 147°C/6 s.

Regarding the products of the advanced Maillard reaction, PELLEGRINO *et al.* (2001) proposed using GAP to evaluate the severity of milk sterilization and discriminate between direct and indirect UHT milk (RESMINI *et al.,* 2003). According to these authors, GAP levels up to 3.5-4 μmoles/L and up to 12-13 μmoles/L are expected for direct and indirect UHT milks, respectively, when good manufacturing practices (GMP) are followed. The GAP levels detected (Fig. 3) ranged from 0.3 to 16 μmoles/L for direct UHT milk and 4.5 to 27.8 μmoles/L for indirect UHT milk. These values confirm that UHT milk samples processed under the minimally required time/temperature combinations are currently available on the domestic market, but the presence of overheated milk is increasing. PELLEGRINO *et al.* (2001) reported that the level of GAP sharply increases only upon severe heat treatment, when milk protein is already extensively glycosylated and degradation of lactulosyl-lysine takes place. In fact, this marker proved to be very sensitive to improper techno-

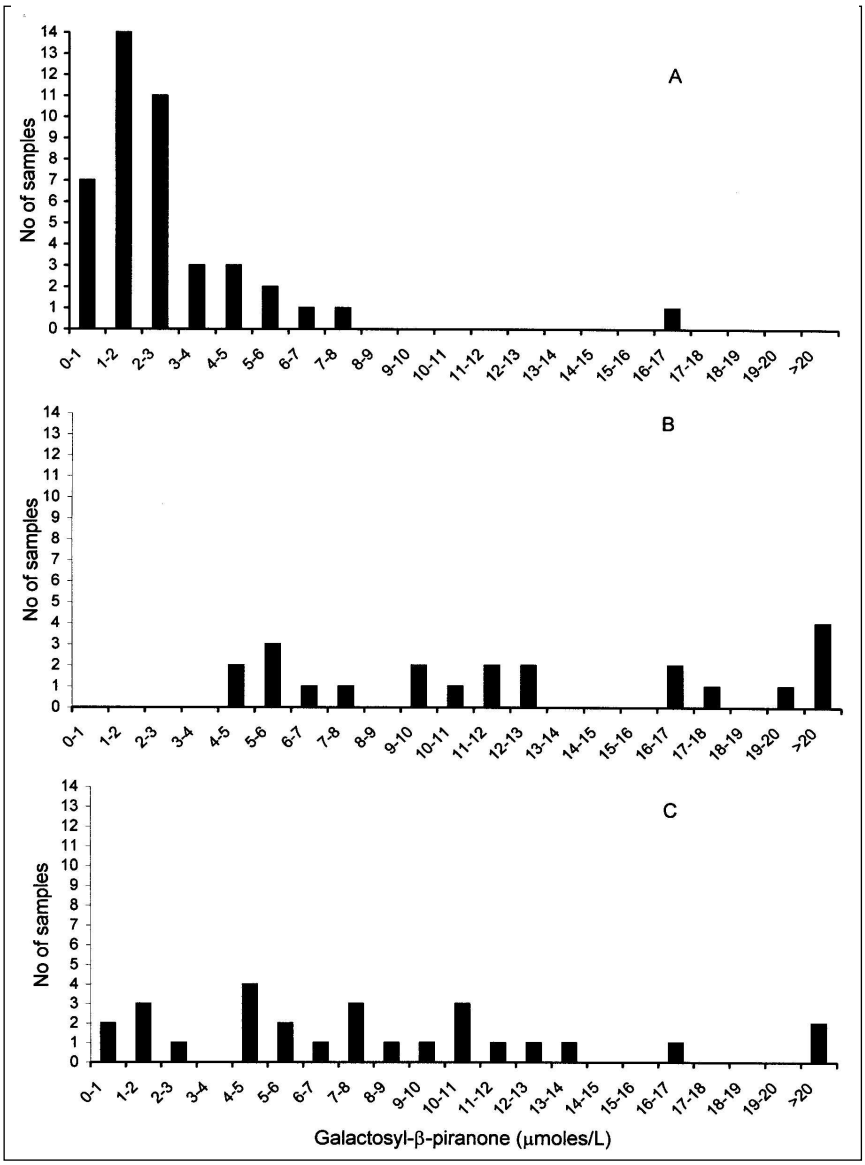

Fig. 3 - Levels of galactosyl-β-piranone in commercial UHT milk samples. Directly (A, n=43) or indirectly (B, n=22) heated and manufactured in Italy, and imported (C, n=27).

logical practices, such as the adoption of a high percentage of milk recirculation in the plant (higher than the usual level of 10%) or even milk re-processing (CATTANEO *et al.*, 2008). In this survey GAP values significantly higher than the upper limits mentioned were clearly identified (Fig. 3) in 9 samples of UHT milk produced in Italy (including 1 direct UHT milk sample). Three samples of imported UHT milk showed the same problem.

It has been demonstrated that the LAL level of UHT milk increases during storage at ambient temperatures. This molecular cross-link has been described as the most sensitive index of the storage period that milk has undergone (CAT-

TANEO *et al.*, 2008). LAL values found in this survey (0.6-5.2 mg/100 g protein for direct UHT milk and 4.6-9.0 mg for indirect UHT milk; Fig. 4) mostly fall within the ranges reported by CATTANEO *et al.* (2008). It should be noted that the highest values for both of these ranges correspond to milk samples that were processed under extreme thermal conditions.

One sample of direct UHT milk and one sample of indirect UHT milk had very high (>14 mg/100 g protein) levels of LAL, even though they were analyzed just a few days after manufacturing. CATTANEO *et al.* (2008) obtained

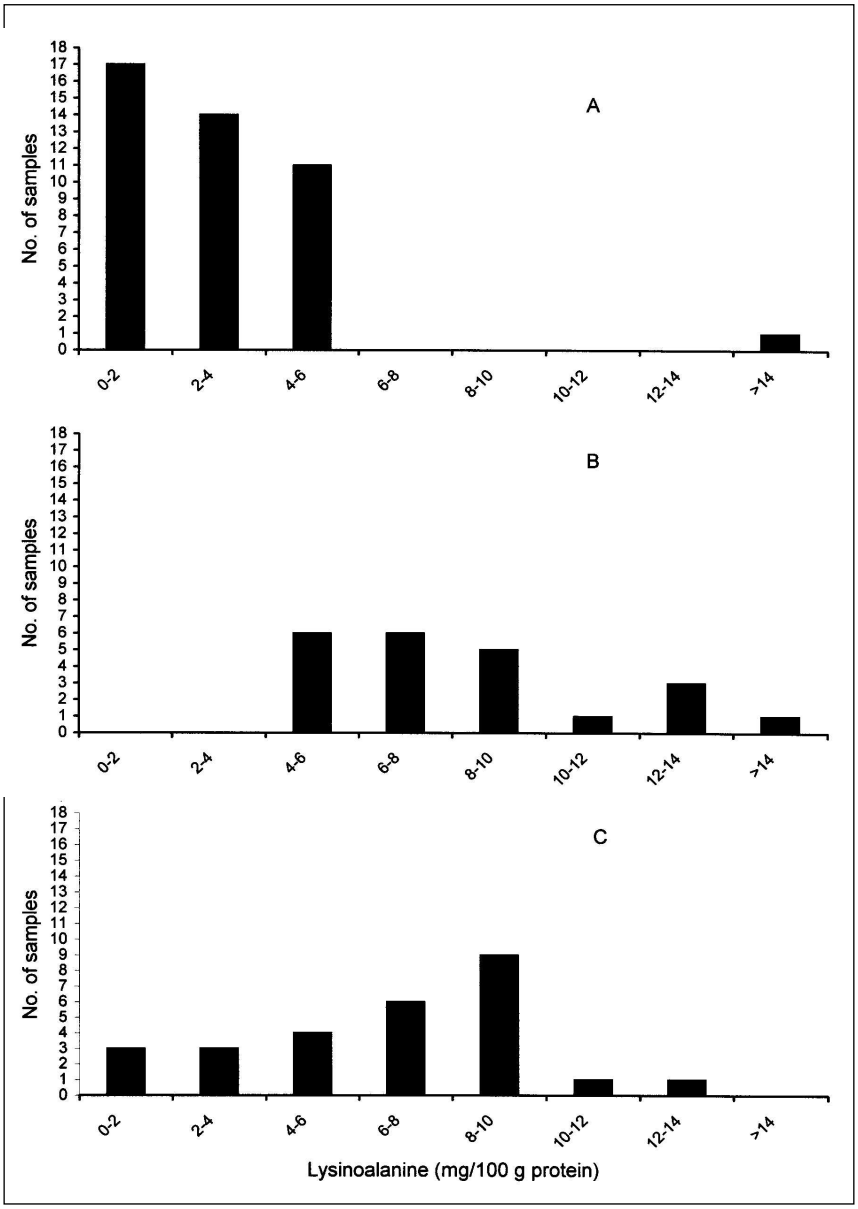

Fig. 4 - Levels of lysinoalanine in commercial UHT milk samples. Directly (A, n=43) or indirectly (B, n=22) heated and manufactured in Italy, and imported (C, n=27).

similar values when indirect UHT milk was manufactured either by adopting a double heat treatment at 148°C/20 s or by re-processing (148°C/20 s) a commercial UHT milk after 90 days of storage at ambient temperature. No imported milk samples showed comparable levels of LAL.

The approach of combining two or more tracers in order to differentiate various thermal treatments of milk has already been proposed (FEINBERG *et al.*, 2006). PELLEGRINO *et al.* (1995) suggested that the level of FUR be coupled with that of LCT in order to characterize sterilized milk. In fact, a close linear relationship exists between these two indices for genuine, freshly prepared UHT milk. The correlation established for the FUR and LCT values for the samples of UHT milk manufactured in Italy are presented in Fig. 5. As a reference, the positioning of 4 samples that were processed under known heating conditions as described by CATTANEO *et al.* (2008) are also shown. Nineteen samples (29%) do not fit the proposed correlation line, falling outside the +2σ confidence limit. According to PELLEGRINO *et al.* (1995) this positioning occurs when the pH value of the starting milk is adjusted before heat processing to improve thermal stability. Even a slight increase of milk pH, with respect to its natural value, promotes a sharp increase in LCT formation upon heat treatment, leaving FUR formation unaffected. Furthermore, in the case of milk overheating, the advanced stage of Maillard reaction is increased and lactulosyl-lysine is converted into the advanced glycosylation end products (AGEs), such as GAP, causing the LCT/FUR ratio to increase and the data point to shift to the left of the correlation line. This effect is clearly seen in Fig. 5; the milk samples falling very far from the +2σ confidence limit (empty symbols, including 2 of the reference samples) show GAP values well above the expected levels mentioned for direct and indirect UHT milk.

It is noteworthy that 5 samples (including 2 direct UHT milk samples) that fit the correlation between LCT and FUR levels have anomalous GAP values. On the basis of their position along the correlation line, the samples are among the most severely treated of the respective types (direct or indirect). The same situation was shown to occur (CATTANEO *et*

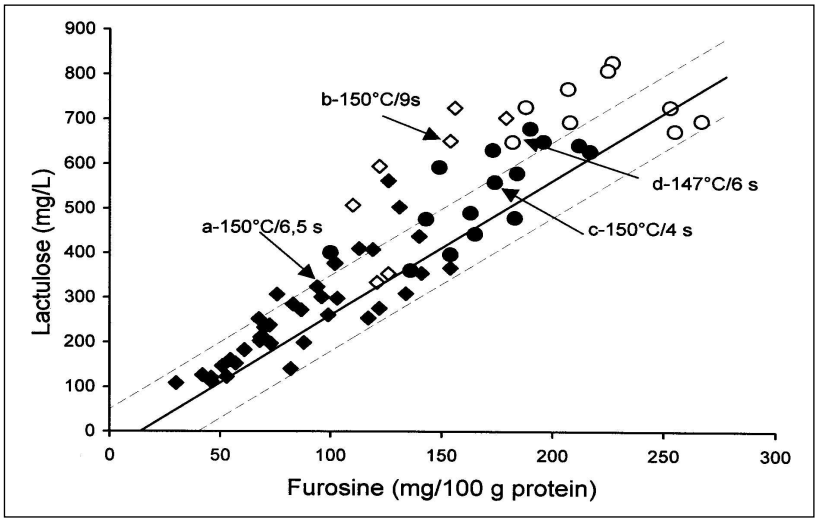

Fig. 5 - Lactulose and furosine in commercial UHT milk samples manufactured in Italy. ♦ = directly heated; • = indirectly heated, empty symbols = samples with anomalous galactosyl-β-pyranone values; a, b, c and d = UHT milk samples from CATTANEO *et al.* (2008). Correlation line (solid line) and ±2σ confidence limits (dashed lines) from PELLEGRINO *et al.* (1995).

al., 2008) when UHT milk was re-processed after prolonged storage. The FUR level increases continuously during storage at ambient temperature, while that of LCT remains more stable and therefore the data point progressively shifts to the right side of the correlation line (PELLEGRINO *et al.*, 1995). The subsequent heat treatment brings an apparently reduced increase of FUR, with respect to LCT, because the advanced Maillard reaction is favored; thus the data point might again fit the correlation, but at a higher level. Therefore, to achieve a complete evaluation of the genuineness of UHT milk, a combined evaluation of the LCT/FUR ratio and GAP level is recommended.

All of the imported UHT milk samples fit the expected relationship between LCT and FUR (Fig. 6), falling within the ±2σ confidence limits of the correlation line, although no distinction could be made between direct and indirect ones. Nevertheless, GAP determination allowed 3 samples to be sorted out that did not respect the characteristic levels of this advanced glycosilation end product for indirect UHT milk. This result suggests unjustified milk overheating or some kind of manipulation, as discussed above.

Overall, 24 samples (37%) UHT milk samples manufactured in Italy and analyzed in this work showed anomalous levels for at least one of the four markers considered. These results indicate the use of operating conditions that cause relevant heat damage. Whether these conditions can be justified needs further discussion. In recent years, the dairy industry has had to face problems in milk sterilization due to the presence of heat-resistant spore-forming (HRS) bacteria, like *Bacillus sporothermodurans* and *Bacillus stearothermophilus*. D_{140} values of 3.4-7.9 s were obtained when a direct UHT sterilizer was used to process contaminated milk (HUEMER *et al.*, 1998). Although these microorganisms are not pathogenic and do not usually alter stability or sensory properties of milk (HAMMER and WALTE, 1996), spores may survive UHT treatment and proliferate in the packaged product during storage reaching unacceptably high levels according to the EU hygiene rules. In order to meet

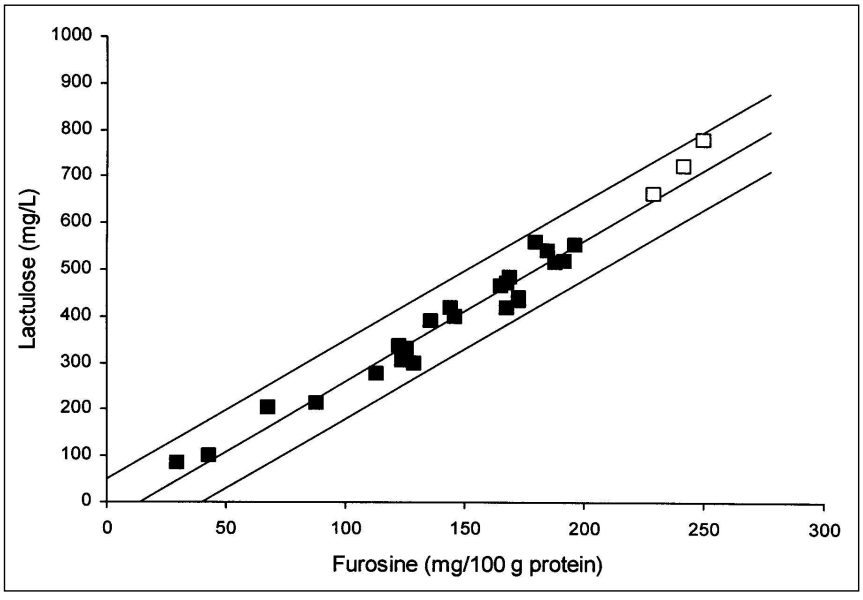

Fig. 6 - Lactulose and furosine in imported commercial UHT milk samples with expected (■) and anomalous (□) galactosyl-β-pyranone values. Correlation line (solid line) and ±2σ confidence limits (dashed lines) from PELLEGRINO *et al.* (1995).

the requested sterility criterion, manufacturers may simply increase the severity of milk processing at their own plant. However, recent knowledge available on this topic (exhaustive review by SCHELDEMAN *et al.*, 2006) should be carefully considered. The most pertinent findings can be summarized as follows: (i) feed concentrate, silage and milking equipment have been identified as possible entry points of spores into raw milk, however the carryover from raw milk to the dairy plant is very limited; (ii) the extreme heat resistance of spores is restricted to particular clones which are probably selected by the heating process; interestingly, a correlation seems to exist between heat resistance of spores and their calcium content; (iii) HRS occurs more frequently in indirect than in direct UHT plants, where they can stick to denatured protein to form biofilms on the surface of pipelines and heat exchangers; and (iv) due to hydrodynamic forces, spores are released into the processed milk and contaminate downstream sections. Consequently, the reprocessing of contaminated lots of UHT milk in the dairy factory is probably the most likely cause of the spread of and contamination by HRS spores in the dairy sector. These observations suggest that the HRS counts may be kept under control by using strategies other than just increasing the severity of milk heat treatments. Among the preventive measures, raw milk of adequate microbiological quality should be used. This aspect is often disregarded in sterilized milk manufacturing. Modern techniques of identifying bacteria by PCR allow raw milk with the lowest levels of HRS (MONTANARI *et al.*, 2004) to be screened rapidly. Another key aspect is the control of fouling because the formation of protein deposits promotes the adhesion of spores (FAILLE *et al.*, 2001). Although fouling cannot be completely eliminated, it can be mitigated by tuning the thermal and hydraulic conditions for the entire plant, not just for the heat exchanger. Although these specific aspects are beyond the scope of this work, in general, increasing the flow rate and decreasing the temperature help reduce fouling.

Some of the samples analyzed in this work had suffered very limited heat damage; this was confirmed for all of the tested parameters. These samples, belonging to both direct and indirect types, demonstrate that the required sterility can be reached without stressing the time/temperature conditions, provided that the whole process is carefully optimized, considering the specifics of the plant in use. GRIJSPEERDT *et al.* (2004) studied the operating conditions in three commercial settings equipped for indirect UHT treatment of milk. They concluded that all systems were over-designed with respect to bacterial inactivation, which have negative effects in terms of product quality and energy utilization.

The top quality direct UHT milk samples were obtained from imported samples. A few samples (1 direct and 4 indirect samples) were found in which the reactions induced by the heat treatment

Table 1 - Levels of furosine (FUR), lactulose (LCT), galactosyl-β-pyranone (GAP) and lysinoalanine (LAL) in some commercial Italian UHT milk samples that show relevant heat damage.

Sample (type of process)	FUR (mg/100 g protein)	LCT (mg/L)	GAP (μmoles/L)	LAL (mg/100 g protein)
A (Direct)	179	704	16.0	16.5
B (Indirect)	225	810	27.8	11.2
C (Indirect)	267	698	24.5	13.8
D (Indirect)	255	675	26.9	17.6
E (Indirect)	253	728	26.9	22.4

reached unacceptable levels. In fact, the values found for the heat damage indices (Table 1) confirm that these samples were manufactured under extreme processing conditions. Comparing these values with those found by CATTANEO *et al.* (2008), it can be stated that the UHT milk samples reported in Table 1 were probably obtained by submitting the milk to two subsequent heat treatments. In fact, GAP levels higher than 16-17 μmoles/L were only reached in indirect UHT milk when (i) milk was processed twice at 148°C/20 s, (ii) a high percentage (>60%) of milk was recirculated in the plant by the diversion valve, or (iii) expired commercial UHT milk was reprocessed at 148°C/20 s. As discussed above, such high GAP levels are found when the Maillard reaction has already taken place extensively during a first heat treatment or after a prolonged storage period. The latter is also responsible for a higher LAL level.

CONCLUSIONS

In contrast to what one would expect regarding the progress of dairy technology and plant engineering, the levels of heat treatment indices in Italian UHT milk are higher than those reported 15 years ago. The processing conditions achieve the sterility requirements of milk but pay little attention to preserving quality. Heat damage reached unacceptable levels in a few samples. Different types of manipulation can be hypothesized for these samples; some are illegal and all are far from the GMP. The presence of such poor quality products on the market should be avoided in order to meet consumer expectations. Today there is a greater awareness of aspects such as milk origin, nutritional value, applied processes and farming systems and hygiene and microbiological safety are taken for granted. The data presented here suggest that the definition of minimal quality requirements for UHT milk can be achieved by fixing an upper threshold for one or more of the heat damage indices reported in this work. This approach is already being used in Italy for pasteurized milk, where threshold levels have been established in terms of soluble whey protein (ITALIAN LAW, 1989). In any case, these indicators would be of value to manufacturers due to their direct applicability in optimizing the processing conditions. They could be systematically measured on a voluntary basis within an in-house quality control program. Furthermore, they would provide consumers with a useful tool for assessing whether milk heat damage was kept within the expected range, thereby preserving quality and taste.

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(1) Manuscripts must be typed, double-spaced and **two** copies submitted along **with** the electronic version. There should be liberal margins on top, bottom and sides (2.5 cm). English is the official language. Authors who are not fluent in written English must seek help from a person fluent in scientific English. The Scientific Editor reserves the right to make literary corrections and to make suggestions to improve brevity, but **the paper must be revised by a native English speaker** before submission.

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The paper must also be submitted by e-mail or on a digital support (cd-rom or floppy disk). Indicate which word processor was used to generate the file and save the file also in format "Text only", DCA-RTF or ASCII, if you do not have programs for Macintosh; **graphics, pictures and diagrams must be saved at 300 dpi in TIF, JPEG, EPS or PICT formats** (not included in MsWord documents).

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