Special Protective Cultures
INTRODUCTION

WHAT IS 4PROTECTION LINE AND WHY USE IT

HOW 4PROTECTION LINE WORKS

4PROTECTION APPLICATIONS:
  • DAIRY
    AYM - ANTI YEAST AND MOULDS
    AL - ANTI Listeria monocytogenes
    AC - ANTI CLOSTRIDIA
    AQSM - ANTI OTHER SPOILAGE MICROORGANISM

STUDIES ABSTRACTS

ABOUT SACCO
No additives, no preservatives, 100% natural are the most prevalent trends that also guide the choices of consumers; safety and durability and high quality standard level of foods is as important as ever. Sacco has the right ingredients for the success of your products and the satisfaction of your customers.

4Protection Special Cultures help to enhance the quality and protect your brand image, allow the product to get to the end of shelf life ensuring a structural and sensorial stability, help to maintain freshness and do not change the taste, aroma and texture. Your ally for a much more genuine product till the consumer table.
Many of the selected strains used were chosen among probiotic microorganisms which has been studied and shown to be effective through specific studies, microbiological tests and sensorial analysis of the products.

Since 1998 Sacco has selected yeasts and bacteria for protection against spoiling unwanted microorganisms in dairy products such as yogurt, fermented milk, fresh cheese, semi-hard cheese, meat and fish. The cultures of 4Protection Lines help to control and preserve the final product from alterations, fighting in a completely natural way any possible unwished bacteria and thereby maintaining a “clean label” product.
The selected 4Protection ferments do not acidify, nor alter the organoleptic characteristics of the product and are easily adapted even at refrigeration temperatures.

Today it is known that microorganisms produce a diverse range of microbial defense molecules including exotoxins, lytic agents, metabolic by-products and bacteriocins (from EFFCA position PFC-2016).

The process is based on a competitive effect for space against microorganisms in general, including pathogens, on the production of anti-microbial metabolites such as organic acids and peptides with specific mode-of-action.

The different applications are studied as a function of the characteristics of the technological process and of the desired performance of the products. Sacco’s technologists are committed to working alongside our customers to find the best solutions and production process, working together with clients offering a product and a customized service.

4Protection line is compatible and complementary to all the Sacco’s starter cultures, they are used by direct inoculation or surface treatment.

Sacco is glad to help customers in finding the best solutions for their specific purpose, according with the characteristics of the products, the technological process and the activity needed from the use of our protective cultures.

HOW 4PROTECTION LINE WORKS

Today it is known that microorganisms produce a diverse range of microbial defense molecules including exotoxins, lytic agents, metabolic by-products and bacteriocins (from EFFCA position PFC-2016). The process is based on a competitive effect for space against microorganisms in general, including pathogens, on the production of anti-microbial metabolites such as organic acids and peptides with specific mode-of-action.
The 4Protection Line helps to improve the products quality and the brand image, reducing non-compliant products, food waste and therefore business costs.

Sacco has 4 lines of products dedicated to the protection of dairy:

- **Anti indigenous yeasts and moulds** AYM
- **Anti Listeria monocytogenes** AL
- **Anti Clostridia** AC
- **Anti Other Spoilage Microorganisms** AOSM
AYM ANTI YEAST AND MOULDS

Protection AYM allows products to reach the end of their shelf life, ensuring structural and sensorial stability, helps to maintain their freshness and does not change their taste, aroma and texture.

<table>
<thead>
<tr>
<th>Product</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPR A</td>
<td>Yogurt, fresh fermented products, fresh cheese, soft cheese, semi hard cheese and hard cheese</td>
</tr>
<tr>
<td>LR B</td>
<td>Yogurt, fresh fermented products, fresh cheese, soft cheese, semi hard cheese and hard cheese</td>
</tr>
<tr>
<td>CLP C</td>
<td>Fresh fermented products, fresh cheese, soft cheese, semi hard and hard cheese</td>
</tr>
</tbody>
</table>

Spoiled yogurt surface percentage during time

Yogurt surface spoiled by moulds after 6 days at 5°C
Inhibition capability of AYM cultures compared to the control.

Challenge test evidences an immediate strong inhibition effect of our LR B while LPR A shows an increasing inhibition effect during time.
**AL – Anti *Listeria monocytogenes***

Protection AL reduces the growth of *Listeria monocytogenes*, increasing the safety of the product throughout its shelf life.

<table>
<thead>
<tr>
<th>Product</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPAL</td>
<td>Soft cheese</td>
</tr>
<tr>
<td>CNBAL</td>
<td>Cheese ripened at low temperature and without sugar, like semi hard and hard cheese, gorgonzola, blue cheese</td>
</tr>
</tbody>
</table>

Counts of *Listeria monocytogenes* in cheese. Day “0” is the day of inoculation with *L. monocytogenes*. The values given are averages of duplicate sampling of three batches. Light blue line indicate low dosage of protective culture 10E6 cfu/g and light green line indicate high dosage 10E7 cfu/g. The culture CNBAL inhibits the growth of *L. monocytogenes*. The higher concentration of the culture, the better inhibition.
**Log10 growth of *Listeria monocytogenes* in samples of cheese**

Counts of *Listeria monocytogenes*, given as log10 cfu/g, in cheese. Day “0” is the day of inoculation with *L. monocytogenes*. The values given are averages of duplicate sampling of three batches.

**Articles and studies:**

- Evidence on inhibition of *Listeria monocytogenes* by divercin V41 action - Richard, Brillat, Pilet, Prévost, Drider (Letters in Applied Microbiology 2003)

- Triton X-114 phase partitioning for the isolation of a pediocin-like bacteriocin from *Carnobacterium divergens* – Métivier, Boyaval, Dufres, Doussot, Compoint, Marion (Letters in Applied Microbiology 2000)

- Delineation of key amino acid side chains and peptide domains for antimicrobial properties of divercin V41, a pediocin-like bacteriocin secreted by *Carnobacterium divergens* V41 – Bhugaloo-Vial, Doussot, Molé, Doussot, Boyaval, Marion (Applied and Environmental Microbiology, 1999)

- Enumeration of *Carnobacterium divergens* V41, *Carnobacterium piscicola* V1 and *Lactobacillus brevis* LB42 by in situ hybridization-flow cytometry - Connil, Doussot, Onno, Pilet, Brout, Montel (Letters in Applied Microbiology 1998)

- Divercin V41, a new bacteriocin with two disulphide bonds produced by *Carnobacterium divergens* V41: primary structure and genomic organization - Méthivier Pilet, Doussot, Sorske, Angladam Zagorec, Prieur, Marion, Cenatiempo, Fremaux (Microbiology 1995)

- Purification and Amino Acid Sequences of Piscicocins V1a and V1b, two class Ila Bacteriocins Secreted by *Carnobacterium piscicola* V1 that display significantly different levels of specific inhibitory activity - Bhugaloo-Vial, Doussot, Métivier, Sorske, Angladam, Boyaval, Marion (Applied and Environmental Microbiology, 1996)
AC – Anti Clostridia

Protection AC acts on Clostridia avoiding the altered aroma, unpleasant smell and ensuring a more consistent and elastic texture and thus a finished product without defects.

<table>
<thead>
<tr>
<th>Product</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC 4P1</td>
<td>Semi soft, semi hard and hard cheese</td>
</tr>
<tr>
<td>LCP 4P2</td>
<td>Smear ripened cheese (typical flavour)</td>
</tr>
<tr>
<td>MO N4P01</td>
<td>Semi soft, semi hard and hard cheese</td>
</tr>
<tr>
<td>MO N4P02</td>
<td>Semi soft, semi hard and hard cheese</td>
</tr>
<tr>
<td>DY 4P13</td>
<td>Semi soft and semi hard cheese</td>
</tr>
</tbody>
</table>

Clostridia control in semi-hard production using LC 4P1

Control

LC 4P1 after 120 days

Raw milk

Pre-coagulation milk (after end of ripening at 120 days 24h at 7°C)
Comparison with lysozyme

Count of spores of Clostridium tyrobutirricum in row milk, pressed curd and final whey with lysozyme (blue histogram) and LC 4P1 (green histogram).

Articles and studies:
- Potential of anticlostridial Lactobacillus isolated from cheese to prevent blowing defects in semihard cheese - Christiansen, Vogensen, Nielsen, Ardö (International journal of dairy Technology 2010).
- Anticlostridial activity of Lactobacillus isolated from semi-hard cheeses - Christiansen, M.H. Petersen, Kosk, Møller, M. Petersen, Nielsen, Vogensen, Ardö (International dairy journal 2005).
Protection AOSM reduces the growth of unwanted indigenous microorganism present in milk or coming from the environment, thus improving the milk storage stability and quality, allowing for a standardization of the production process, in terms of acidification, yield and overall sensory.

AOSM – Anti Other Spoilage Microorganism

<table>
<thead>
<tr>
<th>Product</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>LR B</td>
<td>Raw or pasteurized milk</td>
</tr>
</tbody>
</table>

**Psychrotrophic bacterial growth during milk storage**

**Milk + LR B**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>CFU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>esp1</td>
</tr>
<tr>
<td>24</td>
<td>esp2</td>
</tr>
<tr>
<td>48</td>
<td>esp3</td>
</tr>
</tbody>
</table>

**LR B effect on psychrotrophic bacteria during milk maturation (48h)**

- esp1: -97%
- esp2: -88%
- esp3: -90%
- esp4: -97%
- esp5: -88%
- esp6: -72%
Mesophilic growth during milk storage

Acidification time

LR B effect on mesophilic bacteria during milk maturation (48h)

Acidification time is reduced with maturation of milk with LR B.
Final product sensory

Effect of LR B - 4 production lots average data

Inhibition effect of LR B in a fresh cheese. Reduction of 2-3 log of contaminant.
ABSTRACT

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Evidence on inhibition of *Listeria monocytogenes* by divercin V41 action

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ABSTRACT

C. RICHARD, A. BRILLET, M.F. PILET, H. PREVOST AND D. DRIDER. 2003. Aims: The aim of this study was to investigate the role of divercin V41 in inhibition and prevention of *Listeria monocytogenes*. Methods and Results: *Carnobacterium divergens* V41 deficient in bacteriocin production was isolated and characterized by enzyme-liked immunosorbent assay, multiplex polymerase chain reaction and bacteriocin diffusion test. *Carnobacterium divergens* V41 (divercin+) and *Carnobacterium divergens* V41C9 (divercin–) were grown in the presence of *L. monocytogenes* in smoked salmon model medium. *Carnobacterium divergens* V41, but not *C. divergens* V41C9, was able to inhibit growth of *L. monocytogenes*. The results indicate that inhibition of *L. monocytogenes* in the presence of *C. divergens* V41 is because of the production of divercin V41 and not to a nutritional advantage. Conclusions: *Carnobacterium divergens* V41 may be a promising agent in food safety. Significance and Impact of the Study: The study demonstrates a potential use of a bacteriocin producing lactic acid bacteria in the area food protection. Keywords: *Carnobacterium divergens* V41, divercin V41, class IIA bacteriocin, anti-listerial activity, growth inhibition, food-borne pathogen.
Triton X-114 phase partitioning for the isolation of a pediocin-like bacteriocin from *Carnobacterium divergens*

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2274/99: received 5 August 1999 and accepted 17 September 1999


A new procedure combining Triton X-114 phase partitioning and cation exchange chromatography was developed to purify a bacteriocin from a complex culture medium. This pediocin-like bacteriocin, secreted by *Carnobacterium divergens* and named divercin V41, was entirely recovered in the lower detergent-rich phase whereas all other substances (compounds from culture medium, bacterial metabolites) remained in the upper detergent-poor phase. Subsequent cation-exchange chromatography of the TX-114-rich phase allowed recovery of the pure active bacteriocin and also detergent removing. This new purification method is versatile, fast (only two steps) and can be carried out on whole broth.

**INTRODUCTION**

Increasing consumer interest for food preserved against pathogenic bacteria has led to a considerable increase in the number of scientific papers dealing with bacteriocins, natural antimicrobial peptides and proteins produced by bacteria. Most of these papers have focused on the *in vitro* inhibitory activities, molecular and genetic characterization of new peptides active against the pathogenic *Listeria* strains. Few reports are related to the development of efficient methods compatible with the large-scale production of these active molecules, an essential key for their use to preserve foodstuffs against microbial spoilage.

All bacteriocins have been purified by standard methods with three, four or more steps including ammonium sulphate precipitation, cation exchange or hydrophobic interaction chromatography and C18 reversed-phase HPLC. At the end of purification, the bacteriocin is pure to nearly 100% but peptide recovery is generally low (about 100 mg\(^{-1}\) of culture supernatant). With such small quantities of purified peptide, the characterization of peptide is limited and, in any case, such purification procedures cannot fulfil the quantities required to validate the bacteriocin efficiency in food products.

**ABSTRACT DAIRY**

Anti *Listeria monocytogenes*
Delineation of Key Amino Acid Side Chains and Peptide Domains for Antimicrobial Properties of Divercin V41, a Pediocin-Like Bacteriocin Secreted by \textit{Carnobacterium divergens} V41

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Received 5 August 1998/Accepted 12 April 1999

Divercin V41 (DV41) is a class IIA bacteriocin produced by \textit{Carnobacterium divergens} V41. This antilisterial peptide is homologous to pediocin PA-1 and contains two disulfide bonds. To establish the structure-activity relationships of this specific family of bacteriocin, chemical modifications and enzymatic hydrolysis were performed on DV41. Alteration of the net charge of this cationic bacteriocin by succinylation and acetylation revealed that, in a certain range, the electrostatic interactions were surprisingly not necessary for the activity of DV41. Cleavage of DV41 by endoproteinase Asp-N released two fragments N1[1–17] and N2[18–43] corresponding to the conserved hydrophilic N-terminal and the variable hydrophobic C-terminal sequences, respectively. Inhibitory assays showed that only the C-terminal fragment was active, and after trypsin cleavage at Lys42 or disulfide reduction it lost its inhibitory activity. These results suggested that both hydrophobicity and folding imposed by the Cys25-Cys43 disulfide bond were essential for antilisterial activity of the C-terminal hydrophilic peptide. Chemical oxidation of tryptophan residues by N-bromosuccinimide demonstrated that these residues were crucial for inhibitory activity since modification of any one of them rendered DV41 inactive. On the contrary, only the modification of all the three tyrosine residues caused a total loss of antilisterial activity. These latter results strengthened previous results suggesting that the N-terminal domain containing the YGNGV consensus sequence was not involved in the binding of DV41 to a potential specific receptor on listerial cells.
Divercin V41, a new bacteriocin with two disulphide bonds produced by Carnobacterium divergens V41: primary structure and genomic organization

Anita Métiévier,1,2 Marie-France Pilet,1 Xavier Dousset,1 Odile Sorokine,3 Patricia Anglade,4 Monique Zagorec,4 Jean-Christophe Piard,4 Didier Marion,3 Yves Cenatiempo2 and Christophe Fremaux2,6

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Divercin V41 is a new bacteriocin produced by Carnobacterium divergens V41, a lactic acid bacterium isolated from fish viscera. The amino acid sequence of divercin V41 showed high homologies with pediocin PA-1 and enterocin A. Two disulphide bonds were present in the hydrophilic N-terminal domain and in the highly variable hydrophobic C-terminal domain, respectively. A DNA probe designed from the N-terminal sequence of the purified peptide was used to locate the structural gene of divercin V41. A 6 kb chromosomal fragment containing the divercin V41 structural gene (dvnA) was cloned and sequenced. The results indicate that divercin V41 is synthesized as a pre-bacteriocin of 66 amino acids. The 23-residue N-terminal extension is cleaved off to yield the mature 43-amino-acid divercin V41. In addition, the fragment encodes putative proteins commonly found within bacteriocin operons, including an ATP-dependent transporter, two immunity-like proteins and the two components of a lantibiotic-type signal-transducing system. The genetic organization of the fragment suggested important gene rearrangements.

Keywords: bacteriocin, Carnobacterium, lactic acid bacteria, anti-Listeria

INTRODUCTION

Lactic acid bacteria (LAB) are extensively used in fermented foods not only to improve their flavour and texture, but also to extend their shelf-life. Many LAB are capable of inhibiting growth of some Gram-positive bacteria and pathogenic species such as Listeria monocytogenes, by secreting different antimicrobial compounds, including peptides and proteins. These proteinaceous molecules, also called bacteriocins, have promising potential as food grade preservatives to increase safety of fermented products. The great structural diversity of LAB bacteriocins (Klaenhammer, 1993; Jack et al., 1995) is an opportunity to overcome the problems of resistance, which generally occur with a single bacteriocin, and also provides biotechnologists with different models for the design of new antimicrobial peptides. Among bacteriocin peptides, lantibiotics have been extensively studied and are used as preservatives in some food products (Schillinger et al., 1996). These bacteriocins contain modified amino acid residues, lanthionine and methyllanthionine, which are formed post-translationally (de Vos et al., 1995). Another group of peptides which do not have a modified amino acid residue form a subclass of peptides called the anti-Listeria bacteriocins, class IIa bacteriocins (Klaenhammer, 1993) or cystibiotics (Jack et al., 1995). It

ABSTRACT DAIRY

Anti Listeria monocytogenes
Enumeration of *Carnobacterium divergens* V41, *Carnobacterium piscicola* V1 and *Lactobacillus brevis* LB62 by *in situ* hybridization–flow cytometry

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¹Laboratoire de Microbiologie Alimentaire et Industrielle, Nantes, and ²Station de Recherche sur la Viande, St Genès-Champangelle, France

INTRODUCTION

Flow cytometry (FCM) and epifluorescent microscopy coupled with *in situ* hybridization are new techniques which answer the requirement for rapid and specific enumeration in the food industry, either in microbiological analysis or in the fermentation process.

*In situ* hybridization is a powerful tool for the specific detection of micro-organisms without previous culture (Amann et al. 1996). It has been successfully applied for the detection of *Candida albicans* and *C. tropicalis* with 18S rRNA specific probes. Moreover, *Fibrobacter intestinalis* was detected in pure culture and in mouse caecum samples (Amann et al. 1990a) whereas *Lactococcus lactis*, *Enterococcus faecalis* and *Streptococcus salivarius* were identified in milk in 24 h with specific probes (Beimfohr et al. 1993). The specificity of detection can be increased by the application of multiple probes (Amann et al. 1990a, 1995).

As flow cytometry uses only ‘scatter’ analysis (FSC/SSC; forward light scatter/side light scatter) it is sometimes possible to distinguish two populations in a mixture (Allman et al. 1993; Davey et al. 1996). However, specific detection of bacteria in mixed cultures requires indirect labelling of the cells with a fluorescent marker linked to an antibody or a nucleic probe. Therefore, coupling FCM with *in situ* hybridization may allow rapid enumeration of hybridized micro-organisms. For example, Bertin et al. (1990) used ISH/FCM to detect yeasts, and *Escherichia coli*/*Desulfovibrio gigas* were distinguished in a mixed culture (Amann et al. 1990b); marine nanoflagellates were also enumerated (Rice et al. 1997).

In this context, the present long-term objective is to quantify specifically in cereal, meat or fish products, without culture, useful bacteria such as lactic acid bacteria. For example, some *Carnobacterium* strains (*C. divergens* and *C. piscicola*) which produce bacteriocins should be enumerated among the wild flora of the product or pathogenic bacteria (*Listeria*). The first aim of this study was therefore to enumerate *C. divergens* V41, *C. piscicola* V1 and *Lact. brevis* LB62 in pure culture or in a mixed culture with *L. innocua* F by *in situ* hybridization–flow cytometry.

MATERIALS AND METHODS

Bacterial strains and media

The bacteria used were: *Carnobacterium divergens* V41, *C. piscicola* V1 (Pilet et al. 1994), *Listeria innocua* F (ENITIAA collection) and *Lactobacillus brevis* LB62 (HANSEN strain). *Carnobacterium* strains and *Lact. brevis* LB62 were grown, respectively, in Elliker and MRS broth at 30 °C. *Listeria* innocua F was grown in BHI at 37 °C. The four strains were

ABSTRACT DAIRY

Anti Listeria monocytogenes
Purification and Amino Acid Sequences of Piscicocins V1a and V1b, Two Class IIA Bacteriocins Secreted by Carnobacterium piscicola V1 That Display Significantly Different Levels of Specific Inhibitory Activity

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Received 27 February 1996/Accepted 17 September 1996

Two bacteriocins produced by Carnobacterium piscicola V1 were purified and characterized. Piscicocin V1a (molecular mass = 4,416 Da) and piscicocin V1b (molecular mass = 4,526 Da) are nonlantibiotic, small, heat-stable antibacterial peptides. Piscicocin V1b is identical to carnobacteriocin BM1, while piscicocin V1a is a new bacteriocin. Its complete sequence of 44 amino acid residues has been determined. Piscicocin V1a belongs to the class IIA bacteriocins having the conserved YNGGV motif. These peptides inhibit various gram-positive bacteria, including Listeria monocytogenes. Piscicocin V1a is approximately 100 times more active than piscicocin V1b against indicator strains. However, the antagonistic spectrum is the same for both piscicocins. Comparison of these results with the analysis of the amino acid sequence and secondary structure predictions suggests that (i) the conserved N-terminal conserved domain is involved in the receptor recognition and therefore in an “all-or-none” response against target bacterial cells and (ii) the C-terminal variable and hydrophobic domain determines membrane anchoring and therefore the intensity of the antagonist response.
Potential of anticlostridial Lactobacillus isolated from cheese to prevent blowing defects in semihard cheese

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Five anticlostridial Lactobacillus strains isolated from cheese were selected for a mixed adjunct culture. Cheese with the mixed adjunct culture (experimental) and without (control) was made in triplicate and ripened as vacuum-packed and surface-ripened cheese. Cheese gross composition was similar. Excessive gas formation occurred only in control cheeses. In contrast to control cheeses, the experimental cheeses were dominated by the added adjunct Lactobacillus strains (repetitive-PCR). Casein breakdown was not influenced, however, the total amount of amino acids and pH was slightly lower in the experimental cheeses. Anticlostridial nonstarter Lactobacillus strains have potential as protective adjunct cultures against blowing defects in cheese.

Keywords Cheese, Blowing defects, Clostridium, Lactobacillus, Antimicrobial activity.
Anticlostridial activity of *Lactobacillus* isolated from semi-hard cheeses

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Received 15 April 2004; accepted 9 July 2004

Abstract

Non-starter lactic acid bacteria grow to high numbers in semi-hard cheeses during ripening, and may suppress harmful bacteria. In this study, about 400 *Lactobacillus* isolates from Danish semi-hard cheeses were identified to species level using internal transcribed spacer-polymerase chain reaction (ITS-PCR) analysis. The majority of isolates belonged to the *Lb. paracasei* complex and were classified into approximately 135 types using pulsed field gel electrophoresis (PFGE). *Lactobacillus* isolates representing all the different PFGE types were screened, using an agar well diffusion assay, for antimicrobial activity against 15 single-strain *Clostridium* cultures. Almost half of the isolates possessed anticlostridial activity, and 10% possessed a broad and consistent activity. Nine strains were further investigated for properties of importance for use as mixed cultures in cheese and silage. The results showed that anticlostridial non-starter *Lactobacillus* growing in high quality semi-hard cheeses could be useful as protective adjunct cultures against the growth of *Clostridium*.

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Keywords: Cheese; *Lactobacillus paracasei*; Antimicrobial activity; *Clostridium*
SACCO IS AN INTERNATIONAL COMPANY WITH FAMILY SPIRIT THAT OFFERS A LARGE RANGE OF INNOVATIVE PRODUCTS.

This includes starter cultures for food fermentation (in particular dairy) and nutritional supplements (probiotic cultures), as well as instruments for the improvement of food. The sister company Caglificio Clerici has been an Italian leader in rennet production since 1872. Sacco furthermore acquired the Italian culture producer CSL in 2013. The high quality of our products, the continuous innovation, the ability to work closely with our clients, and the focus on training and developing employees, are the pillars of Sacco. In recent years the company has further invested extensively in R&D, including brand new facilities in 2017, and has been a “pioneer” in areas such as protective cultures. Sacco distributes its products in all key markets (110+ countries), and has ISO 22000 and FSSC 22000 accreditation and a GMP certified plant.

Sacco is a company of Sacco System, the biotech network applied in food, nutraceutical and pharmaceutical industry.
Sacco System

Supporting food culture & life

TRADITION, PASSION
INNOVATION

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