Title: ANTIFUNGAL LACTOBACILLUS PARACASEI STRAIN

Figure 1.10

<table>
<thead>
<tr>
<th>2132</th>
<th>695</th>
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<td>+ Cells</td>
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(54) Title: ANTIFUNGAL LACTOBACILLUS PARACASEI STRAIN

(57) Abstract: The present invention relates to a bacterium of species Lactobacillus paracasei strain DGCC 695 deposited as DSM 28601 at Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures or a functional equivalent thereof.
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ANTIFUNGAL LACTOBACILLUS PARACASEI STRAIN

FIELD OF INVENTION

The present invention relates to a Lactobacillus paracasei bacterium and the use thereof for producing fermented food products or feed products, such as fermented milk-based products, and the use thereof for controlling the growth of at least one contaminating microorganism in a food product or feed product and for preserving a food product or a feed product. The present invention further relates to the use of compounds as antifungal agents and the use of the compounds for preserving a food product or a feed product.

BACKGROUND

Selected bacterial cultures are used on an industrial scale for biopreservation of fresh fermented dairy products. The selected cultures provide efficient inhibition of fungi (e.g. yeasts and/or molds) and serve as an alternative to the use of purified or synthetic antimicrobials. Despite the fact that numerous compounds have been identified as antifungal based on a strategy of bioassay guided fractionation, the factors for antifungal effect remain unexplained. Lack of understanding about the mechanism(s) responsible for the antifungal effect restricts development of new cultures with antifungal properties as well as the application into other food matrices.

In 2009 the yearly global loss and waste of food was estimated to be 1.3 billion tons (1.3 x 10^12 kg). This accounts for approximately one third of the amount produced for human consumption. Focusing only on dairy products, more than 10% of the more than 700 million tons produced milk is wasted per year (Gustavsson et al. 2011). The loss occurs throughout the supply chain from agricultural production to the end-consumer.

Food preservation is one of the means to reduce food waste. Historically, spontaneous fermentation has been one of the means to preserve foods, e.g. by lactic acid bacteria fermentation of milk to yoghurt. Although the high acidity and reduced water activity restricts spoilage by other bacteria, fresh fermented products remain vulnerable towards fungal contamination (Schwenninger, Meile, and Lacroix 2011). Contamination may occur during processing and products will spoil over time, and faster in the case of temperature abuse.
Industrially, synthetic or isolated antifungal agents like benzoic acid and sorbic acid are used for controlling fungal contaminations (Chipley 2005; Stopforth, Sofos, and Busta 2005). As an alternative, food preservation by naturally occurring, selected lactic acid (LAB) and propionic acid bacteria (PAB) has proven to be an interesting possibility. Such bacterial cultures are considered as bio-preservatives (Lacroix 2011; Schniirer and Magnusson 2005).

Cultures with antifungal properties are used on an industrial scale and are marketed by several companies - e.g. DuPont (www.dupont.com); Chr. hansen (www.chr-hansen.com); Handary (www.handary.com); and Clerici Sacco (www.saccosrl.it). These cultures are primarily used in fresh fermented dairy products.

These cultures provide for inhibition of yeast and molds and are added together with the starter culture for yoghurt i.e. requires no extra processing. Cultures with antifungal properties provide an alternative to synthetic antifungal chemical. The use of the cultures with antifungal properties may eliminate the need for synthetic antifungal chemical.

Co-cultures based on combinations of LAB and PAB demonstrate strong antifungal effect in dairy systems (Suomalainen and Mayra-Makinen 1999; Schwenninger and Meile 2004).

The antifungal effect of a co-culture based on Lactobacillus paracasei (LAB A) and Propionibacterium freudenreichii subsp. shermanii (PAB A) is illustrated in figure 1.1a. The figure shows yoghurts stifled with agar prepared without (reference) and with increasing dosages of the co-culture and intentionally contaminated on the surface with two strains of Penicillium. After six days of incubation at 25°C, the growth of the mold on yoghurt with the co-culture was significantly less than on the reference yoghurt.

Although effective in a food system this co-culture shares the problem of a number of antifungal bacterial cultures. Although the biological effect is well-documented, the underlying mechanisms have not been fully elucidated. Antimicrobial activity is the combined effect of numerous factors like competition for nutrients, changes in pH and water activity, change in redox environment and production of bioactive metabolites.

Previous studies typically focused on a strategy of bioassay-guided isolation for the identification of the (individual) compounds considered responsible for the effect.
In case of one active compound and preferably prior knowledge of the compound class, this approach is effective, but it may/will fail for combined effects. Efforts have been made, e.g. for co-cultures similar to the combination of LAB A and PAB A, but hypotheses and applied analytical approaches have not yet provided the answer (Schwenninger and Meile 2004; Schwenninger et al. 2008; Suomalainen and Mayra-Makinen 1999).

The present inventors have developed and applied a chromatography mass spectrometry based metabolomic footprint workflow to investigate the mechanisms behind the antifungal properties of the co-culture consisting of Lactobacillus paracasei (LAB) and Propionibacterium freudenreichii subsp. shermanii (PAB) (e.g. LAB A and PAB A).

The present inventors investigated the effect of the composition of exometabolome of the co-culture on mold growth. A biological model system was developed by the inventors in order to have a simplified system for studying the growth of bacteria and the subsequent effect on mold growth.

Characterization of mold growth was performed by a spectral clustering algorithm on data from multispectral imaging. Untargeted analysis of the exometabolome was performed on LC/MS instrumentation and a combination of methods selected based on expected compound classes in the exometabolome. In order to extend the coverage of the exometabolome, low molecular weight and volatile compounds were analyzed after pre-derivatisation or headspace sampling by GC/MS.

Data from the untargeted LC/MS analysis was processed using feature selection and subsequent multivariate data analysis.

Production of diacetyl occurred without any perturbation of the system by mold hyphae. Presence of LAB cells was required to maintain a diacetyl concentration sufficient for the antifungal effect.

Over time, the concentration of diacetyl decreased and mold developed similar to an acidified uninoculated medium. Besides diacetyl, production of lactic acid and other 2-hydroxy acids contributed weakly to the antifungal effect.

The effect of diacetyl on mold growth in a yoghurt food system was verified. Additionally, relatively higher amounts of diacetyl were produced in yoghurt with than in one without the culture with antifungal properties, indicating that diacetyl also is a major contributor to the effect in food systems.

Comparative analysis of three Lb. paracasei strains demonstrated that the cell-free ferments only possessed a weak inhibitory effect on mold growth. The three strains could be classified according
to their exometabolome and their relative inhibitory effect towards molds. Based on the study, three known and three non-previous reported metabolites were identified as having antifungal properties.

In conclusion, the inventors demonstrated the potential of untargeted analysis of the exometabolome in combination with multivariate data analysis for building new understanding of biological phenomena in food.

STATEMENTS OF INVENTION

Yeast and molds play a major role in spoilage of different types of fermented food products (e.g. dairy products, like yogurt, sweetened and sour cream and fresh and ripened cheese types) and thus can lead to high economic losses. Chemical preservatives like organic acids and their salts (e.g. sorbate and propionate) are used to preserve fermented food or feed products and protect and prolong the shelf life. Drawbacks in using chemical preservatives are the labelling requirements (often as E numbers) and potential adverse effects on the sensory properties of the food product or feed product.

The present inventors identified bacterial strains which surprisingly have antifungal properties. In addition, the present inventors identified compounds which surprisingly have antifungal activity. These bacteria and these compounds are suitable for the preservation of food products and feed products. The bacteria improve the shelf life of food products or feed products produced using the bacteria and/or comprising the bacteria. The compounds improve the shelf life of food products or feed products comprising the compounds. There are times when the sensory perception (e.g. appearance, odour and/or flavor) of a food product or feed product is spoiled before the shelf-life has expired; this spoilage can be at the retailer and/or at the consumer after opening. The bacteria protect the shelf life of food products or feed products produced using the bacteria and/or comprising the bacteria; in other words, the bacteria prevent the sensory perception of the food products or feed products being spoiled before the shelf-life has expired. The compounds protect the shelf life of food products or feed products comprising the compounds; in other words, the compounds prevent the sensory perception of the food products or feed products being spoiled before the shelf-life has expired.

The present invention relates to a bacterium of species *Lactobacillus paracasei* strain DGCC 695 - deposited as DSM 28601 at Leibniz Institute DSMZ-German Collection of Microorganisms and Cell
Cultures GmbH, Inhoffenstraße 7B, 38124 Braunschweig, Germany - or a functional equivalent thereof.

In another aspect, the present invention relates to a combination of bacteria comprising the bacterium according to the present invention and a further bacterium.

In a further aspect, the present invention relates to an inoculum comprising a bacterium according to the present invention or a combination according to the present invention.

The present invention, in another aspect, relates to a culture medium comprising a bacterium according to the present invention or a combination according to the present invention.

The present invention, in a further aspect, relates to a food product or a feed product comprising a bacterium according to the present invention or a combination according to the present invention.

In one aspect, the present invention relates to the use of a bacterium according to the present invention, or a combination according to the present invention, or an inoculum according to the present invention or a culture medium according to the present invention for the production of a fermented milk-based product.

In a further aspect, the present invention relates to a method of producing a fermented milk-based product, comprising contacting a bacterium according to the present invention, or a combination according to the present invention, or an inoculum according to the present invention or a culture medium according to the present invention, with milk or a component of milk.

In another aspect, the present invention provides a fermented milk-based product obtained or obtainable by the method according to the present invention.

In one aspect, the present invention relates to the use of a bacterium according to the present invention, or a combination according to the present invention, or an inoculum according to the present invention or a culture medium according to the present invention for the production of a fermented food product or feed product.

In another aspect, the present invention relates to a method of producing a fermented food product or feed product, comprising contacting a bacterium according to the present invention, or a
combination according to the present invention, or an inoculum according to the present invention or a culture medium according to the present invention, with a substrate for the food product or the feed product.

5 In another aspect, the present invention provides a fermented food product or feed product obtained or obtainable by the method according to the present invention.

The present invention provides, in another aspect, the use of a bacterium according to the present invention, or a combination according to the present invention, or an inoculum according to the present invention or a culture medium according to the present invention, to control the growth of one or more contaminating microorganisms (such as a bacteria and fungi (e.g. yeasts and/or molds)) in a food product or a feed product.

The present invention provides, in a further aspect, the use of a bacterium according to the present invention, or a combination according to the present invention, or an inoculum according to the present invention or a culture medium according to the present invention, for preserving a food product or a feed product.

In another aspect, the present invention provides a culture medium for the identification of metabolites produced by a bacterium according to the present invention or a combination according to the present invention wherein said culture medium comprises a bacterium according to the present invention or a combination according to the present invention.

In a further aspect, the present invention provides a process for the identification of metabolites produced by a bacterium according to the present invention or a combination according to the present invention wherein said process comprises the step of culturing a culture medium comprising a bacterium according to the present invention or a combination according to the present invention and, optionally, isolating the metabolite(s).

In another aspect, the present invention relates to a metabolite obtained or obtainable by culturing a culture medium comprising a bacterium according to the present invention or a combination according to the present invention.

In a further aspect, the present invention provides the use of the compound 2-hydroxy-3-(1 H-indol-3-yl)propanoic acid as an antifungal agent.
In another aspect, the present invention provides the use of the compound 2-hydroxy-3-methylbutanoic acid as an antifungal agent.

The present invention provides, in a further aspect, the use of the compound 2-hydroxy-(4-methylthio)butanoic acid as an antifungal agent.

The present invention provides, in a further aspect, use of a combination of

(a) a compound selected from the group consisting of:

- 2-hydroxy-3-(1H-indol-3-yl)propanoic acid,
- 2-hydroxy-3-methylbutanoic acid, and
- 2-hydroxy-(4-methylthio)butanoic acid; and

(b) another antifungal compound;

as an antifungal agent;

the another antifungal compound of part (b) is, for example, at least one compound selected from the group consisting of:

- 2-hydroxy-4-methylpentanoic acid;
- 2-hydroxy-3-phenylpropanoic acid;
- 2-hydroxy-3-(4-hydroxyphenyl)propanoic acid;
- 2-hydroxy-3-(1H-indol-3-yl)propanoic acid;
- 2-hydroxy-3-methylbutanoic acid; and
- 2-hydroxy-(4-methylthio)butanoic acid;

said compound being different to that of the compound in part (a).

In another aspect, the present invention provides the use of a compound selected from the group consisting of:

- 2-hydroxy-3-(1H-indol-3-yl)propanoic acid,
- 2-hydroxy-3-methylbutanoic acid,
- 2-hydroxy-(4-methylthio)butanoic acid,

and combinations thereof

for preserving a food product or feed product;

optionally said compound is used in combination with another antifungal compound selected from the group consisting of:

- 2-hydroxy-4-methylpentanoic acid;
- 2-hydroxy-3-phenylpropanoic acid; and
2-hydroxy-3-(4-hydroxyphenyl)propanoic acid.

FIGURES

Fig. 1.1a Demonstration of the inhibitory effect of a co-culture of LAB A and PAB A (with LAB:PAB ratio 20:1) in a range of dosages (stated as colony forming units (CFU) LAB A/ml) when co-incubated with yoghurt starter culture at 43°C for 6 hours and then subjected to surface contamination with spores of two strains of Penicillium (Penicillium solitum DCS302 or Penicillium sp DCS1 541) and incubated six days at 25°C.

Figures 1.2 and 1.3 show the microscopic images of LAB prior to inoculation.

Figure 1.4 compares the absorbance of different Lb. Paracasei strains (strains 2132 and 695) after fermentation. Chain length is taken into account.

Figure 1.5 compares the pH after fermentation of different strains of Lb. Paracasei (strains 2132 and 695).

Figure 1.6 examines inoculation according to absorbance.

Figures 1.7a with and without LAB cell presence and 1.7b without LAB cell presence show the inhibition test of different strains of Lb. Paracasei (strains 2132 and 695).

Figure 1.8 shows the influence of the start inoculation on inhibitory activity of cell free ferment of LAB.

Figure 1.9 shows the growth and inhibition of all strains (strains 2132 and 695) when the start inoculation has an absorbance (Abs) of 0.22.

Figure 1.10 shows the inhibition test on all strains (strains 2132 and 695). + cells means that the cells of the LAB strains are present, whereas cell free means that it is only the cell free ferment of the LAB strains.

Figure 1B.1 - Inhibitory effect of compounds #1 to #7 at c1 on Rhodoturola mucilaginosa DCS 1087 at test pH 4.5.

Figure 1B.2 - Inhibitory effect of compounds #1 to #7 at c1 on Rhodoturola mucilaginosa DCS 1087 at test pH 6.0.

Figure 1B.3 - Inhibitory effect of compounds #1 to #7 at c1 on Debaryomyces hansenii DCS 605 at test pH 4.5.

Figure 1B.4 - Inhibitory effect of compounds #1 to #7 at c1 on Debaryomyces hansenii DCS 605 at test pH 6.0.

Figure 1B.5 - Inhibitory effect of compounds #1 to #7 at c1 on Candida sake DCS 1037 at test pH 4.5.

Figure 1B.6 - Inhibitory effect of compounds #1 to #7 at c1 on Candida sake DCS 1037 at test pH 6.0.

Figure 1B.7 - Inhibitory effect of compounds #1 to #7 at c1 on Debaryomyces hansenii DCS 1087 at test pH 4.5.
*Figure 1B.8* - Inhibitory effect of compounds #1 to #7 at c1 on *Debaryomyces hansenii* DCS 1087 at test pH 6.0.

*Figure 1B.9* - Inhibitory effect of compounds #1 to #7 at c1 on *Penicillium spp* - DCS 302 at test pH 4.5.

*Figure 1B.10* - Inhibitory effect of compounds #1 to #7 at c1 on *Penicillium spp* - DCS 302 at test pH 6.0.

*Figure 1B.11* - Inhibitory effect of compounds #1 to #7 at c1 on *Penicillium spp* - DCS 305 at test pH 4.5.

*Figure 1B.12* - Inhibitory effect of compounds #1 to #7 at c1 on *Penicillium spp* - DCS 305 at test pH 6.0.

*Figure 1B.13* - Inhibitory effect of compounds #1 to #7 at c2 on *Debaryomyces hansenii* DCS 605 at test pH 4.5.

*Figure 1B.14* - Inhibitory effect of compounds #1 to #7 at c2 on *Debaryomyces hansenii* DCS 605 at test pH 6.0.

*Figure 1C.1* - The results of the inhibitory properties of the combination of the six compounds (acids) listed in Table 1C.1 (the spot test on surface).

*Figure 1C.2* - Dose response growth curves showing inhibition of growth of *Penicillium* sp. nov. DCS 1541 in the presence of the six compounds (acids) listed in Table 1C.1.

*Figure 1D.1* - Shows examples of growth after three days of two Penicillium strains on cell free fermentations illustrating the inhibitory effect of lab strains.

*Figure 1D.2* - Shows the growth of Penicillium a) DCS 302 and b) DCS 1541 on cell fermentations of the four lab strains.

*Figure 1E.1.* Venn diagram. Metabolite information found in data obtained from analytical platforms is at best both qualitative and quantitative. The identification of metabolites obtained from two platforms might show that some metabolites are shared (qualitative similar), but often a Venn diagram do not state whether the metabolites are quantitatively comparable.

*Figure 1E.2.* A typical covarygram showing the correlation (and covariance) between Standard Normal Variate (SNV) corrected blocks of data (Visual and Near Infrared spectroscopy) and a univariate reference value: the degree of esterification (%DE) [reference 4 of Example 1E]. The black line is the average spectrum, The blue line is the covariance spectrum (scaled to an absolute maximum intensity of 1) and the red line is the Pearson correlation spectrum. Modified from reference 4 of Example 1E.

*Figure 1E.3.* Example of the inadequate use of single metabolites to differentiate between two classes in the experimental design for example a bioactive food product (blue bars and dots) and a reference standard diet (yellow bars and dots).

*Figure 1E.4.* Correlation studies by PCA in an LC-MS study. The loading plot is a simulated plot of shared and unique metabolites extracted from XCMS, MarkerLynx and MZMine, respectively (metabolite names are not included). The highlighted metabolites are shared but they show different correlation patterns which is not represented in e.g. a Venn diagram. In the univariate
correlation plots the real data for two selected metabolites are shown. This highlights the
importance of combining Venn diagrams with multivariate and univariate correlations studies to
discover e.g. an often seen problem for MarkerLynx with too many zeros in the data. More
information about the study design can be found in reference 3.1 of Example 1E.

**Figure 1E.5.** The use of different scaling techniques. Left: No scaling, Middle: Pareto scaling and
Right: Unit variance scaling.

**Figure 1E.6.** Predicted versus measured plot from a PLS model using # principal components -
PC (e.g. PC#4) of creatinine levels using different normalization and scaling techniques. (A-C)
Perdeuterated 3-(trimethylsilyl) propionate sodium salt (TSP) normalized spectra predicting
creatinine concentration using three different scaling techniques; (D-F) 2-norm normalized spectra
predicting creatinine concentration using three different scaling techniques; (G-I) Volume
normalized spectra predicting total creatinine excretion; The points have been colored according to
increasing creatinine concentration/excretion; where cyan corresponds to low concentration and
magenta corresponds to high creatinine concentration/excretion.

**Fig. 1F.1** Growth curves of *Lb. paracasei* DGCC 2132 at 37 °C in MRS and CDIM. Mean values are
average of triplicate determinations. Error bars show the standard deviations.

**Fig 1F.2** Growth of *D. hansenii* DCS 605 and ft. *mucilaginosa* DCS 1063 in CDIM and malt extract
broth (MEB). OD₆₀₀ is measured for 4 at 25 °C. Mean values are average of triplicate
determinations. Error bars show the standard deviations. Upper left: ft. *mucilaginosa* DCS 1063
spotted on MEA (a) and solid CDIM (b) medium in the concentration, 10⁵, 10⁴, 10³ cfu mL⁻¹ after
incubation at 25 °C/3 days. Triplicate determinations (vertical).

**Fig. 1F.3** Growth of *P. solitum* DCS 302 (a and b) and *Penicillium* DCS 1541 (c and d) on MEA (a
and c) and CDIM (b and d) after incubation at 25 °C/4 days.

**Fig. 1F.4** Antifungal activity of a co-culture of *Lb. paracasei* DGCC 2132 and *P. freudenreichii*
subsp. *shermanii* DGCC 2053 in CDIM and milk after fermentation at 37 °C/22 hours. Reference
plates of CDIM and milk without bacteria (a and b) and solidified ferment of CDIM and milk (c and
d) were spotted with *P. solitum* DCS 302 and incubated at 25 °C/4 days. Below images: The
average diameter (mm) of the growth of mold on plates ± standard deviations.

**Fig. 1F.5** Growth of *P. solitum* DGCC 302 on solidified CDIM ferment of *Lb. paracasei* strains with
marked antifungal (DGCC 2132) or little antifungal (693 and 477) effect in milk products after 4
days of incubation of plates at 25 °C. Below images: The average diameter (mm) of the growth of
mold on plates ± standard deviations.

**Fig. 1F.6** Total Ion Chromatograms (top) and Density view of chromatograms (below) of ferments
of *Lb. paracasei* DGCC 2132 and *P. freudenreichii* subsp. *shermanii* DGCC 2053 in UHT milk (left)
and CDIM (right) in reversed phase UPLC in positive ESI MS.
Fig. 1F.7 ESI negative total ion chromatograms (TIC) of milk (top) and CDIM (bottom) fermented with *Lb. paracasei* DGCC 2132 showing ease of detection of 2-hydroxy acids upon a simple dilute and shoot sample preparation strategy in the CDIM (compound labeled 1: 3-(4-hydroxyphenyl)lactic acid; 2: 3-phenyllactic acid).

Fig 1G.1 Schematic biochemical pathway for diacetyl and acetoin in lactic acid bacteria modified from (von Wright & Axelsson, 2011). Enzymes marked in bold, ALDC: alpha-acetolactate decarboxylase, AR: acetoin reductase.

Fig 1G.2 Diacetyl formation and pH change during initial fermentation of *Lb. paracasei* DGCC 2132 in CDIM at 37 °C. pH was measured continuously in 5 replicates while diacetyl was measured during fermentation in two replicates.

Fig 1G.3 a) Diacetyl content in agar plugs of C-ferment and CF-ferment, b) growth of *P. solitum* DCS 302, c) and *Penicillium* sp. DCS 1541 (average ± standard deviation, n=3) in REF (i.e. CDIM at pH 4.5), C-ferment and CF-ferment plates over days. Next to graphs: Images of mold growth on day 4. Day 4 is marked on the graphs by dotted rectangles. Plates were incubated at 25 °C.

Fig 1G.4 Influence of lactic acid and diacetyl (0, 10, 45 and 60 μg/mL) on growth of *P. solitum* DCS 302 (top) and *Penicillium* sp. DCS 1541 (bottom) on CDIM after 4 days of incubation at 25 °C.

Fig 1G.5 400 μg/mL ALDC was added to CDIM prior to inoculation with *Lb. paracasei* DGCC 2132 to decrease formation of diacetyl. C-ferment (a) and C-ferment with added ALDC (b). The influence of added ALDC on inhibition of *P. solitum* DCS 302 was tested after fermentation at 37 °C/24 hours. Plates were incubated at 25 °C until mold growth appeared.

Fig 1G.6 Total ion chromatogram of headspace of yoghurt fermentations after 14 hours at 37°C with added *Lb. paracasei* DGCC 2132 (top) and without *Lb. paracasei* DGCC 2132 (bottom). Peak annotations are 1: CO₂, 2: Acetaldehyde, 3: Dimethylsulfide, 4: Acetone, 5: 2-butanone, 6: 2-pentanone, 7: Diacetyl, 8: Ethanol, 9: 2,3-pentadione, 10: 2-heptanone, 11: Acetoin, 12: 2-Nonanone, 13: Acetic acid, 14: Propanoic acid, 15: Butanoic acid, *: System peaks.

Fig 1G.7 Influence of 0, 45 and 75 μg/mL diacetyl added to yoghurt on growth of *P. solitum* DCS 302 (top) and *Penicillium* sp. DCS 1541 (bottom). Images were recorded after 4 days of incubation at 25 °C.

Fig. 1H.1 Base peak chromatogram of CF of LAB C showing electrospray positive (with insert of enlargement of retention time 4.0 - 7.0 min) and negative (inverted) mode with annotation of selected peaks.

Fig. 1H.2 PCA plot of biological replicates (normalized with IS, pareto scaled) of the three Lb. paracasei strain ferments showing PC1 and PC2 scores (top) and loadings (below) for ESI pos (left) and ESI neg data (right). Identified features designated with abbreviations and unidentified as mass to charge at retention time are listed in Table 1H.7.
Fig. 1H.3 Relative responses of nutrients in the CDIM (REF) and three *Lb. paracasei* CFs showing almost complete depletion of glucose and glutamine in LAB C but only limited consumption of other nutrients. (Sum of responses from adducts and in-source fragments; average ± standard deviation, n=5.)

Fig. 1H.4 Growth after three days at 25°C of mold a) DCS 302 and b) DCS 1541 on CDIM modified to simulate the three *Lb. paracasei* ferments (SIMLAB A, B and C) without and with addition of the six 2-hydroxy acids (n = 3).

Fig. 1H.5 Catabolism of amino acids (AA) with the focus on transamination of amino acids for formation of hydroxy acids in LAB. Enzymes are marked in red: BcAT, ArAT, AspAT, branched-chain-, aromatic- and aspartat aminotransferase; GDH, glutamate dehydrognase, HycDH, hydroxy acid dehydrogenase, KdcA: alpha-keto decarboxylase. Adapted from reference [27] of Example 1H.

Fig. 1H.S1 Growth at 25°C of indicator mold strains a) *Penicillium* sp. DCS302 and b) *Penicillium* sp. DCS1541 spotted on plates of REF (un-inoculated CDIM acidified to pH 4.5) and cell free ferments of LAB A, B and C. (average ± standard deviation, n=3).

Fig. 1H.S2 Pareto scaled PCA scores and loadings of PC1 versus PC2 for ESI neg data (top, a) + b)) and ESI pos data (top, c) + d)) for un-inoculated medium (REF), *Lb. paracasei* ferments (LAB A, B and C) and pooled control samples (MIX).

Fig. 1H.S3a PLSR Model for *Penicillium* sp. DCS 302, day 3 scores plot a), latent variable 2 versus 2 loadings b), predicted versus observed mold growth c) and VIP scores d). Loadings from Pos variables are marked with green stars, and Neg variables are marked with red triangles.

Fig. 1H.S3b PLSR Model for *Penicillium* sp. DCS 1541, day 3 scores plot a), latent variable 2 versus 2 loadings b), predicted versus observed mold growth c) and VIP scores d). Loadings from Pos variables are marked with green stars, and Neg variables are marked with red triangles.

Figure 1J.1. The workflow for quantifying mold growth by multispectral images. The outputs of the analysis allow different samples to be quantified and discriminated.

Figure 1J.2. The layout of the designed Graphical User Interface (PCluster).

Figure 1J.3. Two examples of mold colonies and the corresponding clustered images (clustered images are slightly magnified by the software).

Figure 1J.4. Sample RGB images of the ferments from the chemically defined medium, spotted with *Penicillium* sp. DCS 1541 (left) and milk medium, spotted with *Penicillium glabrum* DCS 305 (right).

Figure 1J.5. Results of analyzing the images from the ferments of the chemically defined medium, a) size of the green and white segments of the colonies (in pixels unit); b) average spectra of the green and white segments.
Figure 1J.6. Sizes of the green segments of the mold colonies (in pixels unit) for the acidified un-
inoculated chemically defined medium samples. a) Penicillium sp. DCS 1541; b) Penicillium solitum DCS 302.

Figure 1J.7. Results of analyzing the images of the milk-based medium ferments. a) size of the green and white segments of the colonies (in pixels unit); b) average spectra of the green and white segments.

Fig. 3.1 General schematics of the omics organization with the flow of information is from genes to transcripts to proteins to metabolites and hence to function or phenotype (from Goodacre 2005 with permission).

Fig 3.2 An illustration showing the principal differences between the analytical technologies, NMR, GCMS and LCMS in terms of sensitivity (M: moles x L^{-1}) and number of metabolites to be detected or identified. Adapted from (Wishart 2013) with permission from Cambridge University Press.

Fig. 3.3 Schematics of the workflow in untargeted metabolomics with some explanatory key words (QC: quality control; MVA: multivariate data analysis). Modified from Hendriks et al. 2011.

Fig. 3.4 Schematics of Microbial interactions through cell-cell contact (1,2 and 4), and contact-independent factors (3, 5-10). From Phelan et al. 2012 with permission from Nature Publishing Group.

Fig 4.1 Schematics of the strategy to correlate metabolic footprints of variations of LAB fermentations and the responding variations in mold growth corresponding to decreasing inhibitory effect from A to C.

Fig. 4.2 Schematics for generating samples (for chemical analysis) and biological response (mold growth) for a set of bacterial conditions (A, B and C). Data acquired during fermentation and incubation are listed aside arrows. Please note ferments are cooled and mold growth assay is performed with and without bacterial cells, whereas only cell-free ferments are stored for chemical analysis.

Fig 5.1 pH profiles (average, n=5) of batch fermentations at 37°C of un-inoculated media (REF) and three Lb. paracasei strains (left) and CINAC data acquisition equipment with 2 x 15 pH electrodes (right). The growth phases are indicated for the LAB B and C strains.

Fig. 5.2 Examples of effect of media composition on morphology of Penicillium solitum (DCS 302) grown a) on CDIM acidified with lactic acid from pH 6.5 to pH 4.0 for three days at 25°C and b) on LAB A ferments with (C) and without cells (CF) compared to CDIM acidified with lactic acid to pH 4.5 (REF) for five days at 25°C (RGB photos generated from multispectral images).

Fig. 5.3 Variation in growth of Penicillium solitum DCS302 a) on different days versus pH (green pixels) and b) versus presence (C) and absence of LAB A cells (CF) (standard deviation indicated, n=3). Dashed rectangles corresponds to time points for RGB pictures shown in Fig. 5.2
Fig. 5.4 Base peak chromatograms of RPC (left) and HILIC (right) in ESI positive (top) and negative (below) RPC conditions (the conditions and instruments used are detailed in Example 1H). HILIC conditions: Injection of 10 μl (5x diluted in MeCN), BEH Amide at 35°C, MP A: 900/50/50 MeCN/H₂O/200 mM NH₃FA pH 3.5 v/v/v; MP B: 950/50 Water/200 mM NH₃FA pH 3.5 v/v; Flow: 300 μl/min; Gradient (time/%B): 0/0, 15/90, 15.5/90, 15.6/0, 25/0-new injection. The LC/MS analysis was performed using an Agilent (Agilent Technologies, Waldbronn, Germany) modular 1290 ultra performance liquid chromatography (UPLC) instrument coupled to a Bruker (Bruker Daltonics, Billerica, MA) maXis 4G single quadrupole time-of-flight mass spectrometer (MS) via an electrospray interface. The UPLC was mounted with a Waters (Waters Corporation, Milford, MA, USA) HSS T3, 2.1 x 150 mm id column packed with 1.8 μm particles. Mobile phases were A) water/formic acid 1000/1 v/v and B) acetonitrile/formic acid 1000/1 v/v. Vials were kept at 5°C in the autosampler prior to injection of 10 μl. Elution was performed with a flow of 400 μl/min and a gradient starting at 0% B at t=0 and kept for 1.0 min, then to 100% B at 15 min and kept for 0.5 minutes. Then back to 0% B over 0.1 minutes and maintained for 4.4 minutes. The electrospray interface with nebulizer at 2.5 bar and dry gas at 9.0 L/min at 200°C was operated in both positive and negative mode (capillary voltage at 4000 V and 3200 V, respectively). Mass spectra in the range m/z 60 - 1250 were acquired with a frequency of 3 Hz. Spectra were saved as centroided. The mass to charge axis was calibrated with sodium formate clusters (solution of water/2-propanol/1 mol/L sodium hydroxide/formic acid 250/250/2.5/0.5 v/v/v/v) infused prior to each chromatographic run via a divert-valve-loop setup. The instrument was controlled using Bruker Daltonics microTOFcontrol version 3.1 and acquired data was handled with Data Analysis version 4.0 SP4.

Fig. 5.5 Illustration of organization of injection sequence with an initial set of QC-old samples for basic system assessment, then a set of equilibrating QC-sample injections followed by randomized brackets of test samples and QC samples.

Fig. 6.1 Schematics of the data processing part of the metabolic workflow. Adapted from (Boccard, Veuthey, and Rudaz 2010).

Fig. 6.2 Illustration of the data structure of a data set (array), consisting of a collection data files (matrices) which again each consists of a number of spectra (vectors).

Fig 6.3 Illustrations of the some of the results during the steps of the workflow in MZmine2

a) MS-noise filtering with red peaks above threshold and blue filtered out
b) Building of chromatograms of m/z 131.0714 based on consecutive scans
c) Attempted deconvolution into several peaks (not achieved)
d) Overlay of aligned features.
Fig. 6.4 Contour plot showing features assigned in ProfileAnalysis marked with rectangles (top) and extracted ion chromatogram of m/z 132.1422 ± 0.058 (below) showing the chromatographic profile of closely eluting isomers. Notice that only Leu and none of the two related compounds lie and Leu-13C2 was assigned.

Fig. 6.5 Correlation plot of peak areas normalized with internal standard calculated with either ProfileAnalysis (left) or MZmine2 (right) versus targeted integration with QuantAnalysis (Analytes: Adenine (top); Met (middle); Riboflavin, vitamin B2 (below)).

Fig. 6.6 Average ESI pos mass spectrum of the Glc peak (0.99 - 1.15 min) showing the multiple potential features for this retention time window with insert of EIC of the annotated peaks (smoothed EICs, algorithm gauss, width 3.4 s, one cyclus).

Fig. 6.7 Principal component analysis scores plot showing QC sampled marked in purple of principal component 2 versus principal component 1 a) before and b) after IS-normalization based on internal standard 1 (Phe-D3) showing a slight improvement in clustering.

Fig. 6.8 The observed technical variation after mean centered depicted as a) standard deviations and b) relative standard deviations (CV%) of repeated injections of RP ESI pos data QC samples (n=18) and the same data after c) pareto scaling and d) logarithmic transformation (after a 20 offset to eliminate zeros).

Fig. 6.9 Plots of PCA scores (left) and loadings (right) after a) mean centering, b) Pareto c) autoscaling d) log transformation with 20 offset and mean centering (five biological replicates, each represented by average of technical duplicate injections).

Fig. 6.10 Scores plot of RPC ESI neg data a) PC1 vs. PC2 showing tight clustering of MIX (QC pooled samples), b) PC1, c) PC2 and d) PC3 versus run number (IS normalized, pareto scaled). LAB A, B, and C are fermented CDIM and REF is un-inoculated (and fermented) CDIM. CDIM is detailed in in Example 1F.

Fig. 6.11 Scores and loadings of PC2 versus PC1 of a PCA model of RP ESI neg data of the analysis of biological replicates (represented by average of technical duplicates) of three different Lb. paracasei strains showing the grouping of samples in a) the scores plot and b) loadings plot with the corresponding features (pareto scaled).

Fig. 6.12 PLSR of RP-ESI MS data versus mold growth of DCS 302 (sum of pixels) on day 3 showing a) plot of root mean square error of calibration (RMSEC) and cross validation (RMSECV) and b) scatter plot of predicted and measured number of pixels based on a four component model (9 observations and 1119 variables, data also referred to in Example 1H, X group scaled and mean centered, Y mean centered).

Fig. 6.13 Illustration of PLS model measures a) VIP scores, b) regression coefficients and c) SR plotted against retention time for selection of biomarkers (data similar to the data in figure 6.12,
also referred to in Example 1H). Green stars and red triangles are features detected in ESI pos and neg, respectively and same features are labeled in all three plots.

**Fig 7.1** Diacetyl measured in LAB A ferments in a) liquid phase (Replicates: pH, n=5, diacetyl n=2) and in b) agar plates based on ferments sampled after 22 hours of fermentation with (C) and without (CF) bacterial cells. Below graphs of growth of the two indicator molds c) DCS302 and d) DCS1541 on the same LAB A ferments and acidified CDIM (REF) (Replicates for b, c and d; n=3, standard deviation indicated, data also referred to in Example 1G)

**Fig. 7.2** The diacetyl/acetoin pathway in lactic acid bacteria with examples of pathways (shaded) that affects the pool of pyruvate and acetolactate. Chemical reactions shown as dotted lines and enzymatic reactions are shown with full arrows with the enzymes listed in blue: ALDC, alpha-acetolactate decarboxylase; ALS, alpha-acetolactate synthase; AR, acetoin-diacetyl reductase. Modified from (Von Wright and Axelsson 2011) and (Goupil-Feuillerat et al. 1997)

**Fig. 7.3** Headspace measurements of a) diacetyl and b) acetoin during fermentation of yoghurt prepared on skimmed milk and with/without the LAB A showing relatively increased of amounts of diacetyl and acetoin with the presence of LAB A.

**Fig. 7.4** Effect of diacetyl spiked (+45, +75, +200 µg/g) into yoghurt and acidified milk against mold growth after 4 days at 25°C (top DCS 302, below DCS1541), and for comparison, yoghurt co-fermented with +LAB A indicating similar effect of LAB A as diacetyl addition of 45 - 75 µg/g diacetyl (data from Stina Aunsbjerg).

**Fig 7.5** Diacetyl measured during a) liquid fermentation and b) in agar plates for mold growth based on ferments sampled after 20 hours of fermentation. LAB A+ is LAB A fermented with an addition of 1%v/v of PAB A ferment. C and CF correspond to cell-containing and cell-free LAB A ferments, respectively. (Standard deviations indicated in a) for pH, n=5 and in b) n=3).

**Fig. 7.6** PCA (IS corrected and Pareto scaled) scores (left) and loading plots (right) of LAB A (red) and LAB A+ (green) of RPpos (top) and RPneg (below) data showing the clustering corresponding to consumption of nutrients (glucose (Glc), adenosine (AR), adenine (A) and amino acids (Gin, Met, Phe, Trp, Leu+Ile) and formation of 2-hydroxy acids (2-hydroxy C6 acids (OH-Me-Pe) and 2-hydroxy-3-(4-hydroxyphenyl)propanoic acid (OH-(OH-Phe)Pr)) Unpublished results, data acquired and handled according to experimental in Example 1H.

**Fig. 7.7** Schematics of the catabolism of amino acids (AA) with the focus on transamination of amino acids for formation of hydroxy acids in LAB. The flavor forming part of the pathway is shown in shade. Enzymes are marked at the side of the arrows: BcAT, ArAT, AspAT, branched-chain-, aromatic- and aspartate aminotransferase; GDH, glutamate dehydrogenase, HycDH, hydroxy acid dehydrogenases, KdcA: alpha-keto decarboxylase (adapted from (Liu et al. 2008)).
DETAILED DESCRIPTION

*Lactobacillus paracasei*

*Lactobacillus paracasei* is a species of lactic acid bacteria. *Lactobacillus paracasei* are used extensively in the food industry such as starter cultures for dairy products or as probiotics. Without wishing to be bound by theory, *Lactobacillus paracasei* improve digestion and immune function.

Examples of *Lactobacillus paracasei* include *Lactobacillus paracasei* strain DGCC 695 and functional equivalents.

*Lactobacillus paracasei* strain DGCC 695 was deposited by Danisco Deutschland GmbH of Busch-Johannsen-Strasse 1, D-25899 Niebull, Germany under the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the purposes of Patent Procedure at Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures on 25 March 2014 under accession number DSM 28601. Danisco Deutschland GmbH have authorized the applicant to refer to the deposited material in the application and given unreserved and irrevocable consent to the deposited material being made available to the public.

The present inventors surprisingly found that *Lactobacillus paracasei* strain DGCC 695 has antifungal properties and is capable of delaying or reducing or preventing the growth of a fungus in, for example, food products and feed products during production of the food products or feed products and/or during storage of the food products or feed products. Thus *Lactobacillus paracasei* strain DGCC 695 is able to control the growth of one or more contaminating microorganisms such as a contaminating fungus (e.g. *Penicillium solitum*).

The term "functional equivalent thereof as used herein includes mutants or variants of *Lactobacillus paracasei* strain DGCC 695 or strains similar thereto, preferably mutants or variants of a *Lactobacillus paracasei* strain, more preferably mutants or variants of *Lactobacillus paracasei* strain DGCC 695. The mutants or variants of strain DGCC 695 may be obtained or be obtainable from cultures of *Lactobacillus paracasei* strain DGCC 695. The mutants or variants of *Lactobacillus paracasei* strain DGCC 695 may be spontaneously derived mutants or variants obtained by culturing *Lactobacillus paracasei* strain DGCC 695 over time. For example, *Lactobacillus paracasei* strain DGCC 695 could be cultured in a specific culture medium for a food or feed product in order to obtain a mutant or variant of *Lactobacillus paracasei* strain DGCC 695.
which has adapted to grow in the culture medium and/or metabolize one or more substrates in the culture medium more effectively than *Lactobacillus paracasei* strain DGCC 695. Alternatively, the mutants or variants of *Lactobacillus paracasei* strain DGCC 695 may be obtained by inducing mutations by, for example, subjecting a culture of *Lactobacillus paracasei* strain DGCC 695 to radiation or chemicals known to induce mutations (e.g. ethyl methane sulphonyate - EMS).

Functional equivalents of *Lactobacillus paracasei* strain DGCC 695 have an antifungal property. Typically, functional equivalents of *Lactobacillus paracasei* strain DGCC 695 have substantially the same or at least substantially the same antifungal property of *Lactobacillus paracasei* strain DGCC 695 (when cultured under the same culture conditions for a given time period).

The nucleotide sequence of 16S rRNA of gene of *Lactobacillus paracasei* strain DGCC 695 (1477bp) is as follows:

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ATCCTTGTGGCGCGTGCTATAATGCAAGTCGAACGAGTTCTCGTTGATGATCGGTGCTTGCACC
GAGATTCAACATGGAAGTGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCTTAAGT
GGGGGATAACATTTGGAAACAGATGCTAATACCGCATAGATCCAAGAACCGCATGGTTCTTGG
CTGAAAGATGGCGTGTAAGCTATCCTGCTTGGTGATGGAGTACCGGATATCCCGTCTCAG
CTAGTGCTCGAGCCCGGCGGTTAATACCTGAGGCTGACGAGTCCCTGAGCCACATGGGA
AGAGATGCGAAGCAGCGTACTGGTACGGGCGGTCGGTGATATCCCAACCAAGGACCCG
CTAATGGCGGAGCCCGCGGCGGATACGCTTGATGGAAGCTGAGTGAAGCGCCGCGGCT
CTGAAAGATGGCGGAGCCGCGGCGGATACGCTTGATGGAAGCTGAGTGAAGCGCCGCG
CTAATGGCGGAGCCGCGGCGGATACGCTTGATGGAAGCTGAGTGAAGCGCCGCGGCT
CTGAAAGATGGCGGAGCCGCGGCGGATACGCTTGATGGAAGCTGAGTGAAGCGCCGCG
CTAATGGCGGAGCCGCGGCGGATACGCTTGATGGAAGCTGAGTGAAGCGCCGCGGCT
CTGAAAGATGGCGGAGCCGCGGCGGATACGCTTGATGGAAGCTGAGTGAAGCGCCGCG
CTAATGGCGGAGCCGCGGCGGATACGCTTGATGGAAGCTGAGTGAAGCGCCGCGGCT
CTGAAAGATGGCGGAGCCGCGGCGGATACGCTTGATGGAAGCTGAGTGAAGCGCCGCG
CTAATGGCGGAGCCGCGGCGGATACGCTTGATGGAAGCTGAGTGAAGCGCCGCGGCT
CTGAAAGATGGCGGAGCCGCGGCGGATACGCTTGATGGAAGCTGAGTGAAGCGCCGCG
CTAATGGCGGAGCCGCGGCGGATACGCTTGATGGAAGCTGAGTGAAGCGCCGCGGCT
CTGAAAGATGGCGGAGCCGCGGCGGATACGCTTGATGGAAGCTGAGTGAAGCGCCGCG
CTAATGGCGGAGCCGCGGCGGATACGCTTGATGGAAGCTGAGTGAAGCGCCGCGGCT
CTGAAAGATGGCGGAGCCGCGGCGGATACGCTTGATGGAAGCTGAGTGAAGCGCCGCG
CTAATGGCGGAGCCGCGGCGGATACGCTTGATGGAAGCTGAGTGAAGCGCCGCGGCT
CTGAAAGATGGCGGAGCCGCGGCGGATACGCTTGATGGAAGCTGAGTGAAGCGCCGCG
CTAATGGCGGAGCCGCGGCGGATACGCTTGATGGAAGCTGAGTGAAGCGCCGCGGCT
CTGAAAGATGGCGGAGCCGCGGCGGATACGCTTGATGGAAGCTGAGTGAAGCGCCGCG
CTAATGGCGGAGCCGCGGCGGATACGCTTGATGGAAGCTGAGTGAAGCGCCGCGGCT
CTGAAAGATGGCGGAGCCGCGGCGGATACGCTTGATGGAAGCTGAGTGAAGCGCCGCG
CTAATGGCGGAGCCGCGGCGGATACGCTTGATGGAAGCTGAGTGAAGCGCCGCGGCT
CTGAAAGATGGCGGAGCCGCGGCGGATACGCTTGATGGAAGCTGAGTGAAGCGCCGCG
CTAATGGCGGAGCCGCGGCGGATACGCTTGATGGAAGCTGAGTGAAGCGCCGCGGCT
CTGAAAGATGGCGGAGCCGCGGCGGATACGCTTGATGGAAGCTGAGTGAAGCGCCGCG
CTAATGGCGGAGCCGCGGCGGATACGCTTGATGGAAGCTGAGTGAAGCGCCGCGGCT
CTGAAAGATGGCGGAGCCGCGGCGGATACGCTTGATGGAAGCTGAGTGAAGCGCCGCG
CTAATGGCGGAGCCGCGGCGGATACGCTTGATGGAAGCTGAGTGAAGCGCCGCGGCT
CTGAAAGATGGCGGAGCCGCGGCGGATACGCTTGATGGAAGCTGAGTGAAGCGCCGCG
CTAATGGCGGAGCCGCGGCGGATACGCTTGATGGAAGCTGAGTGAAGCGCCGCGGCT
CTGAAAGATGGCGGAGCCGCGGCGGATACGCTTGATGGAAGCTGAGTGAAGCGCCGCG
CTAATGGCGGAGCCGCGGCGGATACGCTTGATGGAAGCTGAGTGAAGCGCCGCGGCT
CTGAAAGATGGCGGAGCCGCGGCGGATACGCTTGATGGAAGCTGAGTGAAGCGCCGCG
CTAATGGCGGAGCCGCGGCGGATACGCTTGATGGAAGCTGAGTGAAGCGCCGCGGCT
CTGAAAGATGGCGGAGCCGCGGCGGATACGCTTGATGGAAGCTGAGTGAAGCGCCGCG
CTAATGGCGGAGCCGCGGCGGATACGCTTGATGGAAGCTGAGTGAAGCGCCGCGGCT
CTGAAAGATGGCGGAGCCGCGGCGGATACGCTTGATGGAAGCTGAGTGAAGCGCCGCG
CTAATGGCGGAGCCGCGGCGGATACGCTTGATGGAAGCTGAGTGAAGCGCCGCGGCT
CTGAAAGATGGCGGAGCCGCGGCGGATACGCTTGATGGAAGCTGAGTGAAGCGCCGCG
CTAATGGCGGAGCCGCGGCGGATACGCTTGATGGAAGCTGAGTGAAGCGCCGCGGCT
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In addition or alternatively, functional equivalents of strain DGCC695 have at least 90%, 95%, 97%, 98%, 99%, 99.5% or 100% identity to the 16S rRNA gene of *Lactobacillus paracasei* strain DGCC 695 (SEQ ID NO: 1).
In some embodiments, functional equivalents of *Lactobacillus paracasei* strain DGCC 695 are capable of delaying or reducing or preventing (inhibiting) the growth of one or more of the same fungi as *Lactobacillus paracasei* strain DGCC 695 (when cultured under the same culture conditions for a given time period). In an additional or alternative embodiment, the functional equivalents of *Lactobacillus paracasei* strain DGCC 695 are capable of delaying or reducing or preventing (inhibiting) the growth of one or more fungi which *Lactobacillus paracasei* strain DGCC 695 does not inhibit (when cultured under the same culture conditions for a given time period).

In one embodiment, the inhibition activity of a functional equivalent of *Lactobacillus paracasei* strain DGCC 695, against a particular fungus, is at least substantially the same as that of strain *Lactobacillus paracasei* strain DGCC 695 (when cultured under the same culture conditions for a given time period). In an alternative embodiment, a functional equivalent of *Lactobacillus paracasei* strain DGCC 695 has a greater inhibition activity against a particular fungus when compared to the inhibition activity of *Lactobacillus paracasei* strain DGCC 695 (when cultured under the same culture conditions for a given time period). For example, a functional equivalent of *Lactobacillus paracasei* strain DGCC 695 may have an inhibition activity against a contaminating fungus (e.g. *Penicillium solitum*) which is at least about 80%, or at least about 85%, or at least about 90%, or at least about 95% or at least about 100%, or greater than 100% of the inhibition activity of *Lactobacillus paracasei* strain DGCC 695 against the same contaminating fungus (when cultured under the same culture conditions for a given time period).

The antifungal activity of *Lactobacillus* strains can be assessed in an agar assay. Milk is acidified with a yogurt starter culture and selected test samples are additionally inoculated with the antifungal strains. Fermentation is done until the desired pH is reached (typically 4.60 - 4.40) and the samples are shaken vigorously to break the curd. The yogurt samples are then blended with a pre-warmed agar solution and shaken gently to get a homogenous mixture of yogurt and agar. The yogurt agar is poured in petri dishes and allowed to set. After solidification, diluted solutions of fungi are applied on the surface. The plates are incubated to allow growth of the fungi and after suitable incubation time the growth of the fungi on the plates without antifungal culture is compared to the growth on the plates with antifungal culture added. The inhibition activity can be calculated as follows:

\[
\text{Inhibition activity}\% = 100 - \left(\frac{\text{Area of fungi on plate with antifungal culture}}{\text{Area of fungi on reference plate}}\right) \times 100
\]
In another embodiment, the delay in growth of a contaminating fungus by a functional equivalent of *Lactobacillus paracasei* strain DGCC 695 is substantially the same as that of strain *Lactobacillus paracasei* strain DGCC 695 (when cultured under the same culture conditions for a given time period). For example, the time taken for a contaminating fungus (e.g. *Penicillium solitum*) to grow to the same extent in a food product or feed product comprising a functional equivalent of *Lactobacillus paracasei* strain DGCC 695 is at least about 80%, or at least about 85%, or at least about 90%, or at least about 95% or at least about 100% or greater than 100% of the time taken for the same contaminating fungus (e.g. *Penicillium solitum*) to grow in a food product or feed product comprising the *Lactobacillus paracasei* strain DGCC 695 (when cultured under the same culture conditions for a given time period and/or when stored under the same conditions for a given time period).

The antifungal activity of *Lactobacillus* strains can be assessed in a challenge study in yogurt. Milk is acidified with a yogurt starter culture and selected test samples are additionally inoculated with the antifungal strains. Fermentation is done until the desired pH is reached (typically 4.60 - 4.40) and the yogurt samples are cooled down and dispatched in sterile beakers. The yogurt samples are inoculated with fungi to assess the antifungal activity.

Growth of yeasts is monitored by cell count determination. Inhibition is visible when outgrowth of yeasts is less in the samples prepared with antifungal cultures compared to a reference sample without antifungal culture added.

Growth of moulds is done by visual inspection. Inhibition is visible when outgrowth of moulds occurs later on the surface of the samples prepared with antifungal cultures compared to a reference sample without antifungal culture added.

In an alternative embodiment, a functional equivalent of *Lactobacillus paracasei* strain DGCC 695 has a greater inhibition activity against a particular fungus when compared to the inhibition activity of *Lactobacillus paracasei* strain DGCC 695 (when cultured under the same culture conditions for a given time period). For example, the functional equivalent of *Lactobacillus paracasei* strain DGCC 695 has an inhibition activity against a contaminating fungus (e.g. *Penicillium solitum*) which is at least about 5%, or at least about 10%, or at least about 15% or at least about 20%, or greater than 20% greater than the inhibition activity of *Lactobacillus paracasei* strain DGCC 695 against the
same contaminating fungus (when cultured under the same culture conditions for a given time period).

In another embodiment, the time taken for a contaminating fungus to grow in a food product or feed product comprising a functional equivalent of _Lactobacillus paracasei_ strain DGCC 695 to the same extent as a food product or feed product comprising a _Lactobacillus paracasei_ strain DGCC 695 is longer than that of strain _Lactobacillus paracasei_ strain DGCC 695 (when cultured under the same culture conditions for a given time period). For example, the time taken for a contaminating fungus (e.g. _Penicillium solitum_) to grow to the same extent in a food product or feed product comprising a functional equivalent of _Lactobacillus paracasei_ strain DGCC 695 is at least about 5%, or at least about 10%, or at least about 15% or at least about 20%, or greater than 20%, longer than the time taken for the same contaminating fungus (e.g. _Penicillium solitum_) to grow to the same extent in a food product or feed product comprising the _Lactobacillus paracasei_ strain DGCC 695 (when cultured under the same culture conditions for a given time period and/or when stored under the same conditions for a given time period).

**Further microorganisms**

In one embodiment, the bacterium _Lactobacillus paracasei_ strain DGCC 695, or a functional equivalent thereof, is combined with at least one further microorganism such as a bacterium. For instance, the bacterium _Lactobacillus paracasei_ strain DGCC 695, or the functional equivalent thereof, is combined with at least one further bacterium.

Examples of a further microorganism include one or more microorganisms selected from the group consisting of yeasts, fungi, and bacteria used in the production of fermented food products (such as fermented dairy products) or feed products (such as silage).

Yeast and moulds which are used for producing a food product or a feed product (e.g. moulds and yeasts used for ripening purposes) are typically used in such high doses that they will not be affected by the bacterium _Lactobacillus paracasei_ strain DGCC 695, or functional equivalents thereof.

Examples of a further bacterium include bacteria used in the production of fermented food products (such as fermented dairy products) or feed products (such as silage).
The further bacterium may be selected from the group consisting of lactic acid bacteria, Propionibacterium, a Bifidobacterium or mixtures thereof.

In one embodiment, the bacterium Lactobacillus paracasei strain DGCC 695 or a functional equivalent thereof is combined with one further bacterium (e.g. a Propionibacterium).

In another embodiment, the bacterium Lactobacillus paracasei strain DGCC 695 or a functional equivalent thereof, is combined with at least two further bacteria. For instance, two further bacteria may be selected from the same genera (e.g. the two further bacteria are different lactic acid bacteria species). Alternatively, two further bacteria may be selected from different genera (e.g. one further bacterium is a lactic acid bacterium and the second further bacterium is a Propionibacterium).

In one embodiment, the bacterium Lactobacillus paracasei strain DGCC 695 or a functional equivalent thereof is combined with at least three further bacteria. For instance, the three further bacteria may be selected from the same genera (e.g. the three further bacteria are different lactic acid bacteria species). Alternatively, the three further bacteria may be selected from different genera (e.g. a lactic acid bacterium species, a Bifidobacterium species and a Propionibacterium species).

Examples of further bacteria can be found in Bergey's Manual of Systematics of Archaea and Bacteria (BMSAB) (published by John Wiley & Sons). This manual is also available at http://www.bacterio.net/

Lactic acid bacteria produce lactic acid as the major metabolic end-product of carbohydrate fermentation.

Examples of lactic acid bacteria include, but are not limited to, Lactobacillus spp., Leuconostoc spp., Pediococcus spp., Lactococcus spp., Streptococcus spp., Aerococcus spp., Carnobacterium spp., Enterococcus spp., Oenococcus spp., Sporolactobacillus spp., Tetragenococcus spp., Vagococcus spp., and Weisella spp.

Examples of Lactobacillus spp include Lactobacillus paracasei, L. acidophilus, L. fermentum, L. brevis, L. gasseri, L. plantarum, L. bulgaricus, L. helveticus, L. reuteri, L. casei, L. jensenii, L. rhamnosus, L. crispatus, L. johnsonii, L. salivarius, L. acetotolerans, L. acidifarinae, L. acidipiscis,

Examples of Propionibacterium include, but are not limited to Propionibacterium freudenrechli subsp. shermanii (PAB), Propionibacterium acidifaciens, Propionibacterium acidipropionici, Propionibacterium acnes, Propionibacterium australiense, Propionibacterium avidum, Propionibacterium cyclohexanicum, Propionibacterium freudenrechli subsp. freudenrechli, Propionibacterium granulosum, Propionibacterium jensenni, Propionibacterium microaerophilum, Propionibacterium propionicum, and Propionibacterium thoenii.

In one embodiment, the Propionibacterium is Propionibacterium freudenrechli subsp. shermanii (PAB).

Examples of Bifidobacterium include, but are not limited to, Bifidobacterium adolescentis, B. breve, B. longum, B. animalis, B. infantis, B. thermophilum, B. bifidum, Bifidobacterium catenulatum, Bifidobacterium pseudocatenulatum, Bifidobacterium angulatum and B. lactis.

In one example, the further bacterium is selected from a lactic acid bacterium, a Propionibacterium or a mixture thereof.
In one example, the further bacterium is selected from a lactic acid bacterium, a *Bifidobacterium* or a mixture thereof.

In some embodiment, the bacterium or combination of bacteria is a cell suspension in a liquid.

In some embodiment, the bacterium or combination of bacteria is freeze-dried.

In some embodiments, the bacterium or combination of bacteria is frozen.

The bacteria referred to herein are viable. As used herein, the term "viable" refers to bacterial cells with the potential to have an active metabolism, to survive, grow or multiply.

**Antifungal properties**

*Lactobacillus paracasei* strain DGCC 695 or a functional equivalent thereof has antifungal properties.

The term "antifungal property" as used herein refers to the ability of *Lactobacillus paracasei* strain DGCC 695 or a functional equivalent thereof to control the growth of one or more contaminating microorganisms. The term "control the growth" as used herein refers to delaying or reducing or preventing the growth of at least one contaminating microorganism (such as a contaminating fungus e.g. *Penicillium solitum*) in, for example, culture media or in, for example, food products and feed products during production of the food products or feed products and/or during storage of the food products or feed products.

In one embodiment, *Lactobacillus paracasei* strain DGCC 695 or a functional equivalent thereof has antifungal properties (i.e. it is active) against a fungus. In another embodiment, *Lactobacillus paracasei* strain DGCC 695 or a functional equivalent thereof has antifungal properties (i.e. it is active) against a mold. In a further embodiment, *Lactobacillus paracasei* strain DGCC 695 or a functional equivalent thereof has antifungal properties (i.e. it is active) against a yeast.

Without wishing to be bound by theory, the antifungal property of *Lactobacillus paracasei* strain DGCC 695 or a functional equivalent thereof is caused by the production by the bacterium of at least one antifungal compound or agent.
The terms "antifungal compound" and "antifungal agent" as used herein refer to a compound or agent which is capable of reducing or preventing the growth of a fungus such as a contaminating fungus in, for example, culture media or food products and feed products during production of the food products or feed products and/or during storage of the food products or feed products.

The term "contaminating microorganism" (e.g. contaminating fungus) as used herein refers to any unwanted and unintentional growth of a microorganism (e.g. fungus) in, for example, food products and feed products during production of the food products or feed products and/or during storage of the food products or feed products. In some instances the contaminating microorganism (e.g. fungus) may cause disease when a food product or feed product containing the contaminating microorganism (e.g. fungus) is consumed; without wishing to be bound by theory the contaminating microorganism (e.g. fungus) may produce one or more toxins causing food poisoning. In addition or alternatively, in some instances the contaminating microorganism (e.g. fungus) degrades or causes deterioration of the food product or feed product such that the food product or feed product has: an unpleasant and unwanted taste; and/or an unpleasant and unwanted mouthfeel; and/or an unpleasant visual appearance; and/or an unpleasant odor.

A contaminating microorganism (such as a fungus) may be introduced during the production of a food product or a feed product. In addition or alternatively, a contaminating microorganism (such as a fungus) may be introduced during the packaging and/or storage of a food product or a feed product. Over time, the contaminating microorganism (e.g. fungus) causes quality of the food product or feed product to become spoiled and in some instances it causes the food product or feed product to be no longer safe for consumption.

In one embodiment, the contaminating microorganism is a fungus.

In some aspects the fungus is a mold.

In some aspects the fungus is a yeast.

Examples of contaminating fungus include *PeniciHium* spp. (such as *PeniciHium brevicompactum*, *Penicillium soilum*, *PeniciHium glabrum*, *PeniciHium corylophilum*, and *PeniciHium roqueforti*), *Aspergillus* sp. (such as *Aspergillus ochraceus*, *Aspergillus parasiticus*, and *Aspergillus versicolor*, *Aspergillus niger*), *Eurotium* spp., *Fusarium* spp., *Candida* spp. (such as *Candida colliculosa*, *Candida famata*, *Candida guillermondii*, *Candida kefyr*, *Candida lambica*, *Candida lipolytica*,
Candida lusitaniae, Candida sake, Candida sphaerica, Candida parapsilosis, Candida pelliculosa, Candida rugosa, and Candida zeylanoides), Debaryomyces spp. (such as Debaryomyces hansenii), Kluyveromyces spp. (such as Kluyveromyces marxianus), Rhodotorula spp. (such as Rhodotorula mucilaginosa), Saccharomyces spp. (such as Saccharomyces cerevisiae, and Saccharomyces servazzii), and Geotrichum spp. (such as Geotrichum candidum).

In one embodiment, the contaminating fungus is Penicillium solitum.

In one embodiment, the contaminating microorganism is a bacteria such as an acid sensitive bacteria.

The reduction or prevention of the growth of a contaminating microorganism (e.g. a contaminating fungus) by another microorganism (such as a bacterium, e.g. Lactobacillus paracasei strain DGCC 695 or a functional equivalent) can be determined by determining the inhibition activity of the contaminating microorganism by comparing (i) the growth of the contaminating microorganism (e.g. a contaminating fungus) in the absence of the another microorganism (e.g. Lactobacillus paracasei strain DGCC 695 or a functional equivalent) with (ii) the growth of the contaminating microorganism (e.g. a contaminating fungus) in the presence of the another microorganism (e.g. Lactobacillus paracasei strain DGCC 695 or a functional equivalent) - when cultured under the same culture conditions for a given time period and/or when stored under the same conditions for a given time period. Inhibition activity is calculated as follows:

\[
\text{Inhibition activity [%]} = 100 - \left( \frac{\text{colony diameter in the presence of the another microorganism}}{\text{colony diameter in the absence of the another microorganism}} \right) \times 100
\]

Any method to document and quantify the mold growth relative to a control can be used to assess inhibition activity.

Inhibition activity may be determined using an agar assay. In addition, or alternatively, inhibition activity can be determined in a food or feed challenge.

Inhibition activity in an agar assay may be determined using the multispectral method. The full area (as the pixel count) is measured and used instead of determining colony diameter.
Growth may be monitored in dispersions in liquid media by means of OD as shown in example in 1B - typically the food or feed is transparent.

A challenge study with intended contamination of the food followed by e.g. cfu measurements by e.g. plate counts over time can assist in determining inhibition efficacy.

Typically, the reduction in growth of a contaminating microorganism (such as a contaminating fungus e.g. *Penicillium solitum*) by *Lactobacillus paracasei* strain DGCC 695 or a functional equivalent thereof is such that there is an inhibition activity of at least about 2%, 5%, 10%, 12%, 15%, 20%, 25%, 30%, 35% or 40%. Figure 5.2 shows the growth on un-inoculated CDIM at pH 4.0-6.5.

In some embodiments, inhibition activity in a cell free media may be compared to inhibition in a media acidified with lactic acid (for example, the pH of the acidified media is 4.5).

The delay in the growth of a contaminating microorganism (such as a contaminating fungus e.g. *Penicillium solitum*) by another microorganism (such as a bacterium e.g. *Lactobacillus paracasei* strain DGCC 695 or a functional equivalent) can be determined by comparing

(i) the growth of the contaminating microorganism (such as a contaminating fungus e.g. *Penicillium solitum*) in the absence of the another microorganism (such as a bacterium e.g. *Lactobacillus paracasei* strain DGCC 695 or a functional equivalent) with

(ii) the growth of the contaminating microorganism (such as a contaminating fungus e.g. *Penicillium solitum*) in the presence of the another microorganism (such as a bacterium e.g. *Lactobacillus paracasei* strain DGCC 695 or a functional equivalent)

- when cultured under the same culture conditions for a given time period and/or when stored under the same conditions for a given time period.

Typically, the delay in growth of a contaminating microorganism (such as a contaminating fungus e.g. *Penicillium solitum*) in a food product or feed product by the another microorganism (such as a bacterium e.g. *Lactobacillus paracasei* strain DGCC 695 or a functional equivalent) is such that the time taken for the contaminating microorganism (such as a contaminating fungus e.g. *Penicillium solitum*) to grow in:

(i) the food product or feed product comprising the another microorganism (such as a bacterium e.g. *Lactobacillus paracasei* strain DGCC 695 or a functional equivalent) or
(ii) the food product or feed product produced using the another microorganism (such as a bacterium e.g. *Lactobacillus paracasei* strain DGCC 695 or a functional equivalent),
to the same extent as the contaminating microorganism in:
(i) the food product or feed product which does not comprise the another microorganism
(such as a bacterium e.g. *Lactobacillus paracasei* strain DGCC 695 or a functional equivalent) or
(ii) the food or feed product produced in the absence of the another microorganism (such as a bacterium e.g. *Lactobacillus paracasei* strain DGCC 695 or a functional equivalent).
is at least 2%, 5%, 10%, 15%, 20%, 25%, 30%, 40% or 50% longer (when cultured under the
same culture conditions for a given time period and/or when stored under the same conditions for a
given time period).

In some embodiments, the shelf life is increased by 7-28 days, such as 7-20 days, such as 7-15
days as compared to the same food or feed product without the non-viable preparation derived
from bacteria of the genus *Propionibacterium* stored under the same conditions.

In one embodiment, *Lactobacillus paracasei* strain DGCC 695 or a functional equivalent thereof is
capable of producing at least one antifungal compound or agent.

Typically, the delay in growth of a contaminating microorganism (such as a contaminating fungus
e.g. *Penicillium solitum*) in a food product or feed product by an antifungal compound or agent as
described herein is such that the time taken for the contaminating microorganism (such as a
contaminating fungus e.g. *Penicillium solitum*) to grow in the food product or feed product
comprising the antifungal compound or agent to the same extent as the contaminating
microorganism in a food product or feed product which does not comprise antifungal compound or
agent is at least 2%, 5%, 10%, 15%, 20%, 25%, 30% or 40% longer (when cultured under the
same culture conditions for a given time period and/or when stored under the same conditions for a
given time period).

Without wishing to be bound by theory, the antifungal property of *Lactobacillus paracasei* strain
DGCC 695 or a functional equivalent thereof is caused by the production by the bacterium of one
or more compounds selected from the group consisting of: 2-hydroxy-3-(1 H-indol-3-yl)propanoic
acid; 2-hydroxy-3-methylbutanoic acid; 2-hydroxy-(4-methylthio)butanoic acid; 2-hydroxy-4-methyl
pentanoic acid; 2-hydroxy-3-phenylpropanoic acid; and 2-hydroxy-3-(4-hydroxyphenyl) propanoic acid.

Without wishing to be bound by theory, the antifungal property of *Lactobacillus paracasei* strain DGCC 695 or a functional equivalent thereof is caused or further enhanced by the production by the bacterium of other metabolites such as lactic acid, diacetyl and acetoin. The types of metabolites produced by *Lactobacillus paracasei* strain DGCC 695 or a functional equivalent thereof depend on the media in which the bacterium is cultured. For instance, in a rich media like milk more metabolites may be produced than in chemically defined media.

Examples of antifungal compounds include, but are not limited to: 2-hydroxy-3-(1H-indol-3-yl)propanoic acid; 2-hydroxy-3-methylbutanoic acid; 2-hydroxy-(4-methylthio)butanoic acid; 2-hydroxy-4-methylpentanoic acid; 2-hydroxy-3-phenylpropanoic acid; and 2-hydroxy-3-(4-hydroxyphenyl) propanoic acid. The structure of each of these compounds is shown in Table 7.1.

In one embodiment, the antifungal compound is selected from the group consisting of 2-hydroxy-4-methylpentanoic acid; 2-hydroxy-3-phenylpropanoic acid; 2-hydroxy-3-(4-hydroxyphenyl) propanoic acid; and combinations thereof.

In one embodiment, the antifungal compound or combination of antifungal compounds is active against a fungus.

In another embodiment, the antifungal compound or combination of antifungal compounds is active against a mold.

In a further embodiment, the antifungal compound or combination of antifungal compounds is active against a yeast.

In one embodiment, the antifungal compound or combination of antifungal compounds is formed *in situ* in a food product or a feed product. For example, the addition of *Lactobacillus paracasei* strain DGCC 695, or a functional equivalent thereof, to the substrates (e.g. ingredients) for producing a food product or feed product enables the formation of at least one antifungal compound during the production of the food product or the feed product. In another example, the addition of *Lactobacillus paracasei* strain DGCC 695, or a functional equivalent thereof, to a food product or
feed product enables the formation of at least one antifungal compound in the food product or feed product.

In one embodiment, the antifungal compound or combination of antifungal compounds is used in combination with one or more other antifungal compounds. Examples of suitable antifungal compounds include polyene antifungals such as amphotericin B, candididin, filipin, hamycin, natamycin, nystatin or rimocidin; imidazoles such as bifonazole, butoconazole, clotrimazole, econazole, fenticonazole, isoconazole, ketoconazole, Miconazole, miconazole, omoconazole, oxiconazole, sertaconazole, sulconazole or tioconazole; triazoles such as albaconazole, fluconazole, isavuconazole, itraconazole, posaconazole, ravuconazole, terconazole or voriconazole; thiazoles such as abafungin; allylamines such as amorolfin, butenafine, naftifine or terbinafine; echinocandins such as anidulafungin, caspofungin or micafungin; other compounds with known antifungal properties such as benzoic acid, ciclopirox, flucytosine (5-fluorocytosine), griseofulvin, polygodial, tolnaftate or undecylenic acid; and combinations thereof.

**Culture medium**

In one embodiment, the microorganisms (e.g. the bacterium of the present invention or the combination of bacteria of the present invention) are grown in the optimal culture medium for said microorganism. Using routine techniques, the optimal culture medium can be determined. In addition, the optimal growth conditions can be determined using routine techniques.

Typically a culture medium comprises a carbohydrate source. A culture medium may also comprise a nitrogen source. Further, a culture medium may also comprise other nutrients such as trace metals and/or vitamins.

Typically when the microorganisms (e.g. the bacterium of the present invention or the combination of bacteria of the present invention) is autotrophic towards, for example, one or more amino acids then the amino acids are typically supplied to the media as proteins, protein hydrolysates (e.g. yeast extract, or skim milk powder) and/or individual amino acids.

The culture medium may be any medium suitable for the production of a food product or a feed product.
In some embodiments, the term "culture medium" as used herein does not refer to the shelf-life of a food product or a feed product.

Typically the culture medium comprises one or more substrates used for the production of a food product or a feed product. Examples of substrates for food products and feed products include, but are not limited to milk, vegetables, fruit, cereal, meat, and fruit juices.

Examples of culture media include, but are not limited to milk, components of milk (e.g. whey or casein), and media comprising components of milk.

The milk may be obtained from a cow, goat, sheep, buffalo, yak, camel, reindeer or combinations thereof.

The bacterium of the present invention or the combination of bacteria of the present invention are contacted with a substrate for the food or feed product. For example, an inoculum (e.g. starter culture and/or a culture with antifungal properties) comprising the bacterium of the present invention or the combination of bacteria of the present invention is admixed with a culture medium comprising the substrate for the food or feed product (e.g. milk or a component of milk), optionally a starter culture (which may or may not comprise *Lactobacillus paracasei* strain DGCC 695, or a functional equivalent thereof) is admixed with a culture medium.

Typically, a culture medium comprises the optimal amounts of salts, vitamins and other nutrients necessary for the microorganism.

Typically the microorganisms are cultured at their optimal growth temperature. The skilled person would have readily been able to determine the optimal temperature at which to culture microorganisms mentioned herein.

In one embodiment the microorganisms are cultured at about 1°C to 42°C, 5°C to 42°C, 10°C to 42°C, 15°C to 42°C, 20°C to 42°C, 25°C to 42°C, 30°C to 42°C, 35°C to 42°C, or 37°C to 42°C.

In one embodiment the microorganisms are cultured at about 15°C, 17°C, 19°C, 20°C, 25°C, 30°C, 35°C, 37°C, 40°C or 42°C.
The microorganisms may be cultured for about 3 to 120 hours, 6 to 120 hours, 12 to 120 hours, 24 to 120 hours, 48 to 120 hours or 96 to 120 hours.

The microorganisms may be cultured for about 3 hours, about 6 hours, about 15 hours, about 24 hours, about 48 hours or about 96 hours.

The time period for which a culture medium is cultured in order to produce a food product or a feed product does not refer to the shelf-life or storage of a food product or a feed product at, for example, 5°C.

A culture medium comprising a microorganism may be stored at about 2°C to 10°C, 2°C to 8°C, 2°C to 6°C, or 2°C to 5°C.

In a preferred aspect, the culture medium is inoculated such that there at least $10^5$ CFU of the bacterium/g or ml of culture medium, $10^6$ CFU/g or ml, $10^7$ CFU/g or ml, $10^8$ CFU/g or ml, $10^9$ CFU/g or ml, $10^{10}$ CFU/g or ml, $10^{11}$ CFU/g or ml, $10^{12}$ CFU/g or ml, or $10^{13}$ CFU/g or ml.

The amount of bacteria used to inoculate a culture medium does not refer to amount of bacteria in the food product or the feed product produced by culturing the inoculated culture medium.

The term "inoculum" as used herein refers to the cells that are used to inoculate a culture medium.

The terms "inoculum" and "starter culture" are interchangeable.

In one embodiment, the starter culture is the culture which is typically used for the production of a food product or feed product. In some embodiments, the starter culture further comprises *Lactobacillus paracasei* strain DGCC 695, or a functional equivalent thereof.

In some aspects, the terms "inoculum" and "culture with antifungal properties" are interchangeable.

The term "culture with antifungal properties" as used herein refers to a culture comprising *Lactobacillus paracasei* strain DGCC 695, or a functional equivalent thereof, which is not a final food product or feed product suitable for consumption but which is added to food products or feed products with the aim of delaying or reducing or preventing the growth of a contaminating
microorganism (such as a contaminating fungus). Optionally, the culture with antifungal properties comprises at least one further bacterium.

The terms "culture with antifungal properties" and "protective culture" as used herein may be interchangeable.

Cultures with antifungal properties are used in the production of food products and feed products.

Typically a culture with antifungal properties is used in combination with a starter culture in order to produce a food product or a feed product. The culture with antifungal properties may be added concomitantly with the starter culture to a culture medium or the culture with antifungal properties may be added before or after the starter culture is added to the culture medium.

In some aspects, the terms "culture medium" and "culture with antifungal properties" are interchangeable.

In some embodiments, the culture medium may comprise at least one conventional agent used for controlling the growth of one or more fungi (e.g. yeasts and/or molds).

Examples of conventional agents for controlling the growth of fungi (e.g. yeasts and/or molds) include, but are not limited to propionate, phenylalanine, natamycin, benzoic acid and sorbic acid.

In one embodiment, the method of producing a fermented milk-based product comprises contacting a bacterium according to the present invention, or a combination of bacteria according to the present invention, or an inoculum according to the present invention or a culture medium according to the present invention, with (i) a milk substrate and (ii) at least one conventional agent for controlling the growth of one or more fungi (e.g. yeast and/or molds).

In another embodiment, the method of producing a fermented food product or feed product comprises contacting a bacterium according to the present invention, or a combination of bacteria according to the present invention, or an inoculum according to the present invention or a culture medium according to the present invention, with (i) a substrate for the food product or the feed product and (ii) at least one conventional agent for controlling the growth of one or more fungi (e.g. yeasts and/or molds).
**Food products/ feed products**

The bacterium or combination of bacteria of the present invention may be used as - or in the preparation of - a food product or a feed product.

An antifungal compound or agent as described herein may be used as - or in the preparation of - a food product or a feed product.

The terms "food product" and "feed product" as used herein are intended to cover all consumable products that can be solid, jellied or liquid.

The term "food product" is used in a broad sense - and covers food for humans as well as food for animals (i.e. a feed). In one aspect, the food product is for human consumption.

The "food product" or "feed product" may be in the form of a solid, jelly, or solution depending on the use and/or mode of administration and/or mode of application.

Typically the "food product" is a fermented food product. Typically the "feed product" is a fermented feed product.

Examples of fermented food products include, but are not restricted to, sourdough, beer, wine, cider, fermented vegetables (such as sauerkraut and kimchi), pickled vegetables, fermented meats (such as fermented sausages), juice blends and fermented dairy products.

Examples of fermented dairy products include, but are not restricted to fermented milk-based products - such as yogurt, drinking yoghurt, frozen yoghurt, sour cream, curdled milk, curd, sour milk, sour whole milk, butter milk, cheese, cheese sauce, cream cheese, cottage cheese, whipping cream, kefir, ice cream, lactic acid bacteria drinks, milk drinks, and beverages comprising whey protein.

The term "milk-based product" as used herein means any liquid or semi-solid milk or whey based product having a varying fat content. The milk-based product can be, e.g., cow's milk, goat's milk, sheep's milk, skimmed milk, whole milk, milk recombined from powdered milk and whey without any processing, or a processed product, such as yoghurt, curdled milk, curd, sour milk, sour whole milk, butter milk and other sour milk products.
The "food product" or "feed product" may be in the form of a solution or as a solid - depending on the use and/or the mode of application and/or the mode of administration.

Examples of fermented feed products include, but are not limited to silage or fermented crops.

In some embodiments, the amount of bacteria in the final food product or feed product is in an amount of at least $10^5$ CFU/g or ml, $10^6$ CFU/g or ml, $10^7$ CFU/g or ml, $10^8$ CFU/g or ml, $10^9$ CFU/g or ml, $10^{10}$ CFU/g or ml, $10^{11}$ CFU/g or ml, $10^{12}$ CFU/g or ml, or $10^{13}$ CFU/g or ml of the food product or feed product.

The term "final food product or feed product" as used herein refers to a product which is suitable for consumption, such as for human or animal consumption.

In one embodiment, the food product is a functional food product. The term "functional food" as used herein refers to a food which is capable of providing not only a nutritional effect and/or taste satisfaction but it also capable of delivering a further beneficial effect to the consumer. Accordingly, functional foods are ordinary foods that have components or ingredients (such as those described herein) incorporated into them that impart to the food a specific functional - e.g. medical or physiological benefit - other than a purely nutritional effect.

Although there is no legal definition of a functional food, most of the parties with an interest in this area agree that they are foods marketed as having specific health effects.

Some functional foods are nutraceuticals. Here, the term "nutraceutical" means a food which is capable of providing not only a nutritional effect and/or a taste satisfaction, but is also capable of delivering a therapeutic (or other beneficial) effect to the consumer. Nutraceuticals cross the traditional dividing lines between foods and medicine.

Surveys have suggested that consumers place the most emphasis on functional food claims relating to heart disease. Preventing cancer is another aspect of nutrition which interests consumers a great deal, but interestingly this is the area that consumers feel they can exert least control over. In fact, according to the World Health Organization, at least 35% of cancer cases are diet-related. Furthermore claims relating to osteoporosis, gut health and obesity effects are also key factors that are likely to incite functional food purchase and drive market development.
In one embodiment, the fermented milk-based product is a probiotic.

As used herein, the term "probiotic" means microbial cell preparations or components of microbial cells with a beneficial effect on the health or well-being of the host. (Salminen S, Ouwehand A. Benno Y. et al "Probiotics: how should they be defined" Trends Food Sci. Technol. 1999:10 107-10).

In one aspect, the probiotic composition is an orally administrable composition of metabolically active, i.e., live and/or lyophilized, or non-viable heat-killed, irradiated or lysed probiotic bacteria. The probiotic composition may contain other ingredients. The probiotic composition can be administered orally, i.e., in the form of a tablet, capsule or powder. Other ingredients (such as vitamin C, for example), may be included as oxygen scavengers and prebiotic substrates (such as these improve the colonization and survival in vivo). Alternatively, the probiotic composition of the invention may be administered orally as a food or nutritional product, such as milk or whey based fermented dairy product, or as a pharmaceutical product.

A suitable daily dose of the probiotic bacteria is from about 1 x 10^3 to about 1 x 10^11 colony forming units (CFU); for example, from about 1 x 10^7 to about 1 x 10^10 CFU; in another example from about 1 x 10^6 to about 1 x 10^10 CFU.

In one aspect, the probiotic composition contains the bacterial species and/or cellular components thereof, as active ingredients, in an amount of from about 1 x 10^6 to about 1 x 10^10 CFU/g, respect to the weight of the composition; for example, from about 1 x 10^8 to about 1 x 10^10 CFU/g. The dose may be of 1g, 3g, 5g, and 10g.

Typically, a probiotic is optionally combined with at least one suitable prebiotic compound. A prebiotic is usually a non-digestible carbohydrate such as an oligo- or polysaccharide, or a sugar alcohol, which is not degraded or absorbed in the upper digestive tract. Known prebiotics include commercial products such as inulin and transgalacto-oligosaccharides.

In one aspect, the probiotic of the present description includes a prebiotic in an amount of from about 1 to about 30% by weight, respect to the total weight composition, (e.g. from 5 to 20% by weight). Carbohydrates may be selected from the group consisting of: fructo- oligosaccharides (or FOS), short-chain fructo-oligosaccharides, inulin, isomalt-oligosaccharides, pectins, xylo-
oligosaccharides (or XOS), chitosan-oligosaccharides (or COS), beta-glucans, arable gum modified and resistant starches, polydextrose, D-tagatose, acacia fibers, carob, oats, and citrus fibers. In one aspect, the prebiotics are the short-chain fructo-oligosaccharides (for simplicity shown hereinbelow as FOSs-c.c); said FOSs-c.c. are not digestible carbohydrates, generally obtained by the conversion of the beet sugar and including a saccharose molecule to which three glucose molecules are bonded.

The terms "preserving" and "preservative" as used herein refer to protecting and prolonging (i.e. improving) the shelf life of food products and feed products (e.g. fermented food products and feed products).

The term "shelf life" as used herein refers to the length of time that a food product or feed product is considered to be of a suitable quality for purchase. The term "shelf life" may refer to the length of time that the food product of feed product maintains the expected taste and/or mouthfeel and/or visual appearance and/or odor.

The desired lengths of time and normal shelf life will vary from food product to food product and from feed product to feed product and those of ordinary skill in the art will recognize that shelf-life times will vary upon the type of food product or feed product, the size of the food product or feed product, storage temperatures, processing conditions, packaging material and packaging equipment.

Without wishing to be bound by theory, advantageously, the bacterium or the combination of bacteria according to the present invention protects the length of the shelf life of the food product or feed product. For example, the shelf life of a food product or feed product produced using the bacterium or the combination of bacteria according to the present invention is protected such that at least 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% of a batch of a food product or feed product is not spoiled before the shelf-life has expired when compared to a batch of the same food product or feed product which has not been produced using the bacterium or the combination of bacteria according to the present invention (when stored under the same conditions).

Without wishing to be bound by theory, advantageously, the bacterium or the combination of bacteria according to the present invention increases (improves) the length of the shelf life of the food product or feed product. For example, the shelf life of a food product or feed product
produced using the bacterium or the combination of bacteria according to the present invention is increased by at least 2%, 5%, 10%, or 15% compared to the same food product or feed product which has not been produced using the bacterium or the combination of bacteria according to the present invention (when stored under the same conditions).

The expiration date or the use-by date of a food product or feed product refers to the safety of the food product or feed product for consumption and/or the length of time that the food product of feed product maintains the expected taste and/or mouthfeel and/or visual appearance and/or odor. After the expiration date, the food product or feed product is no longer considered safe for consumption and/or the expected taste and/or mouthfeel and/or visual appearance and/or odor of the food product or feed product has deteriorated.

A food product or feed product as described herein may be stored at about 2°C to 10°C, 2°C to 8°C, 2°C to 6°C, or 2°C to 5°C.

The present invention is further described by way of the following non-limiting examples.

**EXAMPLES**


**List of abbreviations**
AL  Alpha-acetolactate
ALDC  Alpha-acetolactate decarboxylase
APCI  Atmospheric pressure chemical ionization
AR  Acetoin-diacetyl reductase
C  Cell containing ferment
CDIM  Chemically defined interaction media
CF  Cell free ferment
CFU  Colony forming units
DHS  Dynamic headspace sampling
DGCC  DuPont Global Culture Collection
DCS  DuPont Collection Safety
EI  Electron ionization
EIC  Extracted ion chromatogram
ESI  Electrospray
GC  Gas chromatography
HILIC  Hydrophilic interaction liquid chromatography
HS  Headspace sampling
LAB  Lactic acid bacteria, and with index (A, B and C) to specify *Lactobacillus paracasei* strain and with '+' (as in LAB A+) to show addition of ferment of PAB A
m/z  Mass to charge ratio
MIC  Minimal inhibitory concentration
MS  Mass spectrometry
MVA  Multivariate data analysis
Neg  Negative (as in electrospray negative)
NMR  Nuclear Magnetic Resonance
OD  Optical density
PAB  Propionic acid bacteria, and with index (A) to specify *Propionibacterium* strain
PC, PCA  Principal component, and principal component analysis
PLSR  Partial least squares regression
PPM  Parts per million
Pos  Positive (as in electrospray positive)
QC  Quality control
RGB  Red-green-blue
RP, RPC  Reversed phase (liquid chromatography), and reversed phase chromatography
Rt  Retention time
SHS  Static headspace sampling
SPE  Solid phase extraction
SPME  Solid phase microextraction
TIC    Total ion chromatogram
UPLC  Ultra-performance liquid chromatography
Amino acids are designated according their three letter abbreviation.

DGCC 695 used in the following examples was obtained from Germany.

The term "LAB A" as used herein refers to the lactic acid bacteria *Lactobacillus paracasei* DGCC 2132.

The term "LAB B" as used herein refers to the lactic acid bacteria *Lactobacillus paracasei* DGCC 11287.

The term "LAB C" as used herein refers to the lactic acid bacteria *Lactobacillus paracasei* DGCC 695.

The term "PAB" or "PAB A" as used herein refers to propionic acid bacteria *Propionibacterium freudenreichii* subsp. *shermanii* DGCC 2053.

**EXAMPLE 1A - SCREENING OF LESS INHIBITORY LAB IN CHEMICALLY DEFINED INTERACTION MEDIUM (CDIM)**

A screen for growth and antifungal activity of strains of *Lb. paracasei* in CDIM was carried out to find strains with similar growth patterns as *Lb. paracasei* DGCC 2132 but with a different antifungal activity. Strain *Lb. paracasei* DGCC 2132 is a commercially available strain. CDIM is described in Example 1F.

The present inventors found that *Lb. paracasei* DGCC 2132 aggregates whereas other strains grow as single cells or in short chains thus inoculation according to cfu gives different concentration of cells. Figures 1.2 and 1.3 show the microscopic images of different LAB strains (*Lb. Paracasei* or PABs) prior to inoculation.

Figure 1.4 compares the absorbance of different *Lb. paracasei* strains after fermentation. Chain length is taken into account. The strain may be grown in the presence of a PAB A (1% v/v%).
Figure 1.5 compares the pH after fermentation of different strains of *Lb. paracasei*. A low pH indicates a faster acidification which could give shorter production time of e.g. fermented dairy products.

Figure 1.6 examines inoculation according to absorbance of different strains of *Lb. Paracasei*. CDIM was inoculated with $10^7$ cfu/mL of a LAB which corresponds to an initial absorbance of 0.22. Thus all batches shown in Figure 1.6 were inoculated to obtain an absorbance of 0.22. The strains were then compared by absorbance and pH measurement after 20 hours fermentation at 37°C.

Figure 1.7a shows the inhibitory activity of different LAB strains. The top row (+ cells) shows the ferment with the LAB cells, the row below shows cell free ferments of the LAB strains. The inhibition test was carried out by fermentation at 37°C for 20 hours. The plates were spotted with *Penicillium solitum* (*P. solitum*) 302 (20 µL of $10^5$ cfu/mL) and incubated at 25°C for 4 days. The cell-free inhibition test was carried out by fermentation at 37°C for 3 days; cells were then removed by centrifugation and filtration and plates were spotted with *P. solitum* 302 (20 µL of $10^5$ cfu/mL) and incubated at 25 °C for 4 days.

Figure 1.7b shows cell free ferment after 20 hours fermentation at 37°C and 3 days of incubation at 25 °C. The top row and the bottom row are duplicates. The plates spotted with *P. solitum* 302. The bottom row is a duplication of the top row.

Figure 1.8 shows the influence of the start inoculation on inhibitory activity of cell free ferment. The top row shows a starting inoculation absorbance of 0.22, the row below is a starting inoculation absorbance of 0.1 corresponding to about $2.5 \times 10^6$ cfu/mL of 2132.

Figure 1.9 shows the growth and inhibition of all strains when the start inoculation has an absorbance (Abs) of 0.22. All batches were grown in MRS overnight. The cells were washed and CDIM was inoculated with all strains to obtain an abs =0.22.

Figure 1.10 shows inhibition test on all strains. + cells means that the cells of the LAB strains are present, whereas cell free means that it is only the cell free ferment of the LAB strains. The CDIM is inoculated with bacteria to obtain an absorbance of 0.22. The batches are then incubated at 37°C for 20 hours before plate preparation with or without bacterial cells. *P. solitum* 302 is spotted on dried plates (20 µL of $10^5$ spores/mL).
EXAMPLE 1B - Antifungal screen

Six potential antifungal compounds were screened alone at two concentration levels each and in combination thereof, at two pH levels (4.5 and 6) against four yeast and two mould strains. The inventors determined if the concentrations of the compounds as found in a LAB fermentate were sufficient enough to cause antifungal activity. Furthermore, the pH dependency of the antifungal action was evaluated by testing under acetic environment (pH 4.5), as often found in food application, and neutral conditions (pH 6.0).

Method

Indicator strains were grown overnight.

The test strains used for the evaluation of the antifungals were isolates of different origin e.g. national strain collections (e.g. DSMZ, ATCC) and spoiled food product isolates, etc collected in the in- house DuPont Culture Collection Safety (DCS) (DuPont, Brabrand, DK), maintained at -86°C in suitable broth containing glycerol 50% (v/v). The strains were grown, following a two consecutive cultivation cycle.

In essence, 10 ml of YM broth (Sigma Y3752; Copenhagen, Denmark), suitable for the growth of yeast were inoculated with one colony already grown YGC agar (VWR: AX021259, Denmark) plate and incubated at 25 °C for 48 hours. From this stock culture an overnight culture was prepared by 40x dilution in fresh broth.

For mould strains spores were used as the inoculum. Mould spore inoculums were prepared as follows: One loop of mould (5 day old plate) was diluted in approximately 2 ml of sterile demineralised water and spread on sloping malt extract agar (Merck catalogue no. 1.05398.0500) using a inoculation needle. The tube was incubated at 25 °C for 3 - 5 days. Storage medium (tap water with 0.01% Tween 80, pH adjusted with sulphuric acid) for mould spores was added and the biomass loosened using an inoculation needle. This suspension was transferred to a sterile container.

The overnight cultures and the spore suspensions of each indicator strain (see list below) are diluted to a final inoculation rate of 5.0E+04 CFU/ml.

The following strains were used:
A stock solution with a concentration (cO) of 50g/L of the compounds #1 to #6 (see listed below) was prepared. The stock solution was left to dissolve on the roller mixer (FPV1 1-127). Compounds #3, #4, #6 were heated for 5 sec. in the microwave to dissolve completely. The stock solution is further diluted (2Xc1) in the test media (YM-broth) at the appropriate pHs (4.5 and 6.0). The work stock 2Xc1 is then 100-fold diluted to 2Xc2, with 2Xc2 being 2-times the concentrations found in the LAB fermentate as determined with HPLC.

Compound #7 was prepared by mixing compounds #1 to #6 at the ratio of 1:1:1:1:1:1 either at 2Xc1 or 2Xc2.

The compounds were:

<table>
<thead>
<tr>
<th>#</th>
<th>Code</th>
<th>Concentration</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>A1097</td>
<td>5189 mg/L</td>
<td>2-hydroxy-4-methylpentanoic acid = '2-hydroxyisocaproic acid' (from Leu);</td>
</tr>
<tr>
<td>#2</td>
<td>A1098</td>
<td>4814 mg/L</td>
<td>2-hydroxy-3-phenylpropanoic acid = 'phenyllactic acid' (from Phe);</td>
</tr>
<tr>
<td>#3</td>
<td>A1099</td>
<td>4815 mg/L</td>
<td>2-hydroxy-3-(4-hydroxyphenyl)propanoic acid = '(4-hydroxyphenyl)lactic acid' (from Tyr);</td>
</tr>
<tr>
<td>#4</td>
<td>A1100</td>
<td>5188 mg/L</td>
<td>2-hydroxy-3-(1H-indol-3-yl)propanoic acid = 'Indolelactic acid' (from Trp);</td>
</tr>
<tr>
<td>#5</td>
<td>A1101</td>
<td>5190 mg/L</td>
<td>2-hydroxy-3-methylbutanoic acid = '2-hydroxyisovaleric acid' (from Val);</td>
</tr>
<tr>
<td>#6</td>
<td>A1102</td>
<td>5231 mg/L</td>
<td>2-hydroxy-(4-methylthio)butanoic acid (from Met); Available as Calcium salt;</td>
</tr>
<tr>
<td>#7</td>
<td></td>
<td></td>
<td>Mix of 1-6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>C2</th>
<th>C1</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>65</td>
<td>6500 mg/L</td>
</tr>
<tr>
<td>#2</td>
<td>21</td>
<td>2100 mg/L</td>
</tr>
<tr>
<td>#3</td>
<td>16</td>
<td>1600 mg/L</td>
</tr>
<tr>
<td>#4</td>
<td>4.4</td>
<td>440 mg/L</td>
</tr>
<tr>
<td>#5</td>
<td>23</td>
<td>2.3 g/L</td>
</tr>
<tr>
<td>#6</td>
<td>4.4</td>
<td>0.44 g/L</td>
</tr>
<tr>
<td>#7</td>
<td>134</td>
<td>13.4 g/L</td>
</tr>
</tbody>
</table>
A 96-well plate was filled 95 µl of the cultivation broth, either at pH 4.5 or at pH 6. One hundred microliter of the earlier prepared compound solutions (2X) are added accordingly. Finally the 5 µl of the diluted strain preparation was used to inoculate each well. Each strain and compound combination was added in duplicates. Following the below pattern:

<table>
<thead>
<tr>
<th>CX</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1-pH Y</td>
<td>A</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>#2-pH Y</td>
<td>B</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>#3-pH Y</td>
<td>C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>#4-pH Y</td>
<td>D</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>#5-pH Y</td>
<td>E</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>#6-pH Y</td>
<td>F</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>#7-pH Y</td>
<td>G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>H</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

After inoculation, the microwell plates are incubated at 25 °C for up to 60 hours, with 3 reading points at 0h (t0) and 40h and 60h (t1). Optical density (OD 620nm) was used to detect outgrowth of the indicator strains. The results, OD development over time (Δ OD t1-t0) and the relative inhibition (Δ OD compound (t1-t0)/Δ OD control (t1-t0)) are shown in Tables 1B.1 to 1B.2 and Tables 1B.3 to 1B.6, respectively.

Figures 1B.1 to 1B.12 show the effect of compounds #1 to #7 at c1 (at both test pHs) on different indicator strains. Figures 1B.13 and 1B.14 show the effect of compounds #1 to #7 at c2 (at both test pHs) on Debaryomyces hansenii DCS 605, the only strain that was somewhat susceptible to the lower test concentration. (Remaining figures not shown: growth control and treated strains follow the same growth curve.

**Conclusion**

Sufficient outgrowth of the target organism (without an antifungal compound present) is considered when AOD (t1-t0) reached a value of <0.1. As can be seen in Table 1B.1, DCS 1037 and DCS 605 did not grow to a reliable cell density after 40 hours at both test pHs. Also DCS 302 did not reach the threshold OD of 0.1 at pH 4.5 after 40 hours. Further calculations for these strains after 40 hour incubation should therefore not be considered in the effectiveness evaluation of the compounds. After 60 hour of incubation at 25 °C all indicator strains reached the threshold.

As can be seen in the figures 1B.1 to 1B14, the indicator strains respond differently to the compounds. However, compound #1 and #7 (the blend of compound #1-#6), at a concentration
100 times higher than found in a actual LAB fermentate (see Table 1.C.1), were nearly without any exception the only treatments that caused delayed outgrowth of the indicator strain. Without wishing to be bound by theory, the antifungal activity found in the combination #7 could therefore be attributed to the #1 present in that blend. The effects observed appear to be pH independent. At concentrations found in the fermentate (c2) (no further improvement of specific production) #1 to #6, and even the combination of the compounds demonstrated only very little antifungal effect. DCS 605 was the only strain were also the lower test concentration (c2) caused a suppressed growth behavior of the target.

**EXAMPLE 1C - INHIBITORY EFFECT OF SIX 2-HYDROXY ACIDS DETECTED IN CELLS FREE FERMENTS OF LACTIC ACID BACTERIA**

Example 1C details the observations of inhibitory effect for individual and combinations (ferment simulation) of six 2-hydroxy acids detected in cells free ferments of lactic acid bacteria. Table 1C.1 details the compounds and concentrations used for studies of individual and combined effect.

Table 1C.1 Quantification of identified metabolites in LAB ferments with highest VIP scores in mold growth models (average ± st dev, n=5).

<table>
<thead>
<tr>
<th>Compound#</th>
<th>Compound (abbreviation)</th>
<th>L. paracasei strain</th>
<th>LAB A mg/L</th>
<th>LAB B mg/L</th>
<th>LAB C mg/L</th>
<th>Limit of detection mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2-hydroxy-4-methyl pentanoic acid (OH-Me-Pe)</td>
<td>12.3 ± 0.2</td>
<td>24.5 ± 0.5</td>
<td>65 ± 6</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2-hydroxy-3-phenylpropanoic acid (OH-Phe-Pr)</td>
<td>&lt;2</td>
<td>21.1 ± 0.9</td>
<td>11.6 ± 1.0</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2-hydroxy-3-(4-hydroxyphenyl) propanoic acid (OH-(OH-Phe)-Pr)</td>
<td>&lt;1</td>
<td>15.8 ± 0.7</td>
<td>6.5 ± 0.4</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2-hydroxy-3-(1H-indol-3-yl)propanoic acid (OH-Ind-Pr)</td>
<td>&lt;3</td>
<td>4.4 ± 0.2</td>
<td>&lt;3</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>2-hydroxy-3-methylbutanoic acid (OH-Me-Bu)</td>
<td>5.6 ± 0.1</td>
<td>5.3 ± 0.3</td>
<td>23.4 ± 2.2</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>2-hydroxy-4-(methylthio)butanoic acid (OH-MeS-Bu)</td>
<td>1.2 ± 0.03</td>
<td>4.4 ± 0.2</td>
<td>2.7 ± 0.2</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>Sum of acids used</td>
<td></td>
<td>19</td>
<td>76</td>
<td>109</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Experimental** - Inhibitory properties of the combination of the six 2-hydroxy acids - Spot test on surface.

**Mold Growth Assay** The effect on mold growth was assessed for LAB ferments (C and CF) compared to acidified un-inoculated CDIM acidified to pH 4.5 by titration with lactic acid (REF).
Simulated ferments were used for testing the effect of pH, the presence of 2-hydroxy acids and absence of glucose on mold growth. CDIM was acidified with DL-lactic acid to pH corresponding to average pH of LAB A, LAB B, and LAB C respectively after 3 days of fermentation. Identified and quantified compounds were added in the concentrations produced by the 4 strains (Table 1C.1). Batches corresponding to ferment of LAB C were made with and without glucose. CDIM pH adjusted with DL-lactic acid without added compounds detailed in Table 1C.1. was used as the reference. Batches were made in triplicates.

Mold growth was measured via multispectral images on day 3 of mold growth.

**Results:**

The results of the inhibitory properties of the combination of the six 2-hydroxy acids (the spot test on surface) are shown in Figure 1C.1. The term "glc" refers to glucose.

The term "+ acids" refers to the addition of combination of compounds (2-hydroxy acids) listed in Table 1C.1 at the given pH (Ref, controlled by lactic acid).

Multifactor ANOVA of mold growth by varying the pH and adding 2-hydroxy acids showed that (i) pH (4.6 vs. 3.8) was a significant factor for both strains and(ii) 2-hydroxy acids (with/without) to be significant for strain DCS 1541.

When the experiment is analyzed as a nested design by Variance Component Analysis (main factor pH, secondary, nested factor acids), then pH explained for 75% of the variation for DCS 1541 and 95% DCS 302.

For DCS 1541 the acid factor accounted for ca. 9% - but the residual (noise) for 12% and hence, considered as a nested design the effect of acids is not significant.

**Inhibitory properties of the six 2-hydroxy acids individually and in combination - in solution**

**Experimental:**

The six identified compounds (Table 1C.1) were tested for their minimal inhibitory concentration (MIC) against the two strains of *Penicillium*. Solutions of CDIM acidified with DL-lactic acid (pH 4.5) were prepared with 0 (Reference), 0.1, 1, 5 and 10 mg/mL of all six compounds, respectively. The
Test solutions were distributed into a sterile flat-bottomed 96 well microplate (Fisher Scientific) and 10^4 spores/mL of each *Penicillium* strain were added to the wells. Triplicate determinations were made. Microplates were incubated at 25°C for up to 48 hours.

OD at start (t₀) and after 48 hours (tₜ) was recorded at 600 nm using a Varioskan Flash (Thermo Fisher Scientific Oy, Finland).

The inhibition degree (ID) was calculated and evaluated as (Miescher Schwenninger, J. Food Protection (2008)):

\[ ID = \frac{\text{OD}_{t₀} - \text{OD}_{tₜ}}{\text{OD}_{t₀} - \text{OD}_{t₀}} \]

The minimal inhibition concentration at 50% inhibition (MIC 50) was defined as the concentration providing ID < 0.5 (Miescher Schwenninger, J. Food Protection (2008), Vol. 71, No. 12, pp. 2481 - 2487).

The inhibitory effect was assessed of the combinations of the six 2-hydroxy acids as detected in the four ferments and with/without addition of 10 µg/ml diacetyl which corresponds to the residual amount found in cell free ferments. The acids was dosed as in the experiment for agar plate spot tests, i.e. in concentrations as listed in Table 1C.1.

**Results:**

The results of the inhibition assay (MIC 50, 48 h in solution) are shown in Table 1C.2

Table 1C.2 - Inhibition degree of OH-Me-Pe, OH-Phe-Pr, OH-(OH-Phe)Pr, OH-Ind, OH-Me-Bu and OH-MeS-Bu towards *P. solitum* DCS 302 *Penicillium* spp. DCS 1541 in CDIM acidified with DL lactic acid to pH 4.5 (Average ± standard deviation, n=3). Compounds are added in the concentrations 0.1, 1, 5 and 10 mg/mL. The lowest concentration needed to give MIC 50 is highlighted in bold.

<table>
<thead>
<tr>
<th>Compound (abbreviation)</th>
<th>Concentration (mg/ml)</th>
<th>Inhibition Degree (48 hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>DCS 302</td>
</tr>
<tr>
<td>OH-Me-Pe</td>
<td>0.1</td>
<td>1.07 ± 0.20</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.75 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.36 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.06 ± 0.01</td>
</tr>
</tbody>
</table>
All individual compounds were found to have inhibitory effect, but at concentrations more than 100x higher than found in ferments. The estimated MIC 50 values (highlighted in bold) are in the range 5-10 mg/ml corresponding to literature where available. The combination of 2-hydroxy acids in ferments only provide weak inhibition which improved slightly by the addition of diacetyl.

Conclusions:

A weak but significant inhibitory effect towards *Penicillium* sp. DCS 1541 of the addition of the combination of 2-hydroxy acids corresponding to produced levels at pH 4.5 and 3.8 were observed (spot test).

The most prominent effect was due to pH.

No significant inhibitory effect towards *Penicillium* spp. DCS 302 under the abovementioned conditions was observed (spot test).
All six compounds have weak inhibitory effect with MIC 50 of the individual 2-hydroxy acids towards *Penicillium* sp. are in the range 5 - 10 mg/ml which is more than 100x higher than produced in the ferments (liquid phase) (these findings are in accordance with literature, where available). Compound #4 is more inhibitory than others towards DCS1541.

Only a weak effect was observed of the combination of acids in the MIC 50 study.

A similar study to the above looking at the inhibition of *Penicillium* sp. nov. DCS 1541 in the presence of 2-hydroxy acids (0 (REF), 1, 5 and 10 mg/mL) was performed and the OD measured over a 5 day period. Growth curves showing the dose response are presented in Figure 1C.2. The 2-hydroxy acids in Figure 1C.2 are indicated as follows: 2-hydroxy-4-methyl pentanoic acid (OH-ME-Pe) (#1), 2-hydroxy-3-(1H-indol-3-yl)propanoic acid (Indole) (#4), 3-phenyllactic acid (PLA) (#2), 2-hydroxy-3-methylbutanoic acid (OH-Me-Bu) (#5), 2-hydroxy-3-(4-hydroxyphenyl) propanoic acid (OH-PLA) (#3), 2-hydroxy-4-(methylthio)butanoic acid (OH-MeS-Bu) (#6).

Growth of molds in presence and absence of 2-hydroxy acids may be determined by use of OD and by use of an optical detection system based on time-lapse imaging, such as the oCelloScope™ (marketed by Phillips).

The oCelloScope™ detection system is an instrument where images of the growing fungi can be recorded inside the wells of a microtitre plate. The focus plane of the imaging system is tilted 6.25° and several images are recorded inside the microplate well. By using a segmentation and extraction of surfaces areas algorithm it is possible to determine the growth of the microorganisms from the recorded images.

**EXAMPLE 1D**

Further experimental detail can be found in Examples 1G and 1H.

Figure 1D.1 shows examples of growth after three days of two Penicillium strains on cell free ferments illustrating the inhibitory effect of lab strains.

Figure 1D.2 shows the growth of Penicillium a) DCS 302 and b) DCS 1541 on cell ferments of the three lab strains.
EXAMPLE 1E - Chemometrics goes Foodomics - what’s in the blocks!

With the advent of advanced high throughput analytical platforms for measuring biological samples it has become common to measure the same samples on more than one analytical platform. Foodomics studies are normally concerned with multifactorial problems and it can be useful to explore and measure the same samples on several complementary and synergistic analytical platforms. As these platforms consist of multifactorial sensors and separation methods the data analytical challenge of exploring, extracting and describing the data increases exponentially. Moreover, the risk of becoming flooded with non-informative data ("too much data, too little information") increases concomitantly.

On the other hand acquisition of data from different analytical platforms provides new opportunities for checking the validity of the data, for comparing analytical platforms and for ensuring proper data (pre)processing. All this can be studied in the context of correlation studies. This Example provides some practical and pragmatic tools to validate and deal advantageously with data from more than one analytical platform. The key point is to emphasize the need for complementary correlations studies both within and between blocks of data to ensure proper data handling, interpretation and dissemination. Correlation studies can be regarded as a preliminary step prior to multivariate data analysis or as an introduction to more advanced multiblock methods.

1 Introduction
Foodomics as defined here is more than classical Quality Control (QC) and bulk chemical analysis of food and/or body fluids in relation to food control and nutritional research, and foodomics is more than just the use of advanced analytical platforms for analyzing sample composition. In the following Example concept Foodomics are used to simultaneous analyse many samples using a high throughput analytical platform such as gas/liquid chromatography (GC/LC=XC) followed by mass spectral detection (MS) and/or Nuclear Magnetic Resonance spectroscopy (NMR) which are able to simultaneous detection of hundreds or thousands metabolites in the biological samples [1].

Seen from a physical, chemical and biological perspective food systems (and biofluids) are complex multifactorial systems containing mixtures of heterogeneous classes of molecules as well as complex physical structures such as amorphous solids, aqueous solutions, gels, macromolecules, macroorganelles, cells, crystals and pores and cavities. The complex nature of food samples thus makes extraction and separation on a specific column (GC or LC) combined with a mass separator the preferred choice of analytical method. Other less destructive techniques
such as NMR and vibrational spectroscopy (near-infrared, infrared and Raman) can also be applied, but they cannot match the sensitivity of hyphenated separation and mass spectrometry systems. With the hyphenation of a separation technique and a mass spectrometer (XC-MS) the data output becomes two-dimensional and very large and it becomes more challenging and time-consuming to extract the proper information directly from the raw data.

The data exploration and processing of XC-MS data is further challenged by the presence of artefacts such as irrelevant signals from column components, skewness in data due to mass uncertainties and ion suppression and shifted peaks across samples, column deterioration over time, drift in baseline, injection variations across samples etc. NMR data contain less spectral artefacts, but it is challenged by pH effects, ionic effects and water signal contributions. Preprocessing of data is therefore a significant part of the whole foodomics data analysis chain.

Applications of foodomics include studies of foods for food control, food safety, food functionality, food authenticity, food traceability, food freshness, food contaminants, food toxicity, food bioactivity as well as food effects on human health. Foodomics studies are thus often designed to investigate well-defined scientific questions related to food and food consumption. The problem under investigation can for example be: what causes one diet to be healthier than the control standardized diet - the health beneficial effect may be known, but the mechanism and the responsible metabolites remain elusive. When analyzing data from such studies the target is clear: find the differences in the metabolite profiles that can be related to the experimental design (i.e. the two diets). Even though the question is simple, the complexity and size of foodomics data with several analytical platforms, the information from hundreds or even thousands of metabolites and on top of that the natural biological variation of individuals makes the data exploration nontrivial. Foodomics thus cannot be defined without taking into account the current methodologies for exploring and exploiting patterns and relations in the mega/multivariate data structures that are generated.

Classical chemometric methods and methods originating from different or related research fields are available and can directly be transferred to foodomics. Chemometrics or multivariate data analysis is a powerful method to explore correlations/co-variations in such datasets using only a minimum of a priori assumptions. This can either be done with (supervised) or without (unsupervised) a priori knowledge about the experimental design. The nature of many foodomics studies makes the investigation of correlations between the data blocks an important aspect in not
only the exploration of the data but also in the validation of the data and in the necessary preprocessing of the data.

This Example provides a toolbox of simple methods and a guideline for the data exploration of foodomics studies that include data from several analytical platforms. The Example advocates for a systematic combination and fusion of data blocks to assess the analytical performance and to improve the interpretability and prevent the reporting biased and unreliable results. This Example includes "eye-opener" examples on how to use well-established chemometric techniques in novel ways in order to augment the information content and enhance the understanding of the foodomics data.

2 Correlation studies; possibilities and pitfalls

Correlation studies range from simple univariate comparisons [2, 3] to high dimensional patterns linking many metabolites together through various chemometrics methods. Linking two analytical platforms is often done through a Venn diagram as shown in Figure 1E.1.

The identification of metabolites obtained from two platforms might show that some metabolites are shared (qualitative similar), but often a Venn diagram does not state whether the metabolites are quantitatively similar. For some analytical platforms this is not expected due to ‘out of linear range’, 'major differences in selectivity' etc. whereas for other metabolites the quantitative nature should be comparable. In depth knowledge about the analytical behavior and capabilities of the instrumental techniques will strongly help in avoiding incorrect interpretation of shared metabolites, but without correlations studies the risk of examining non validated (quantitatively) metabolites is large and can hamper the subsequent chemometric models and from this the interpretation of foodomics studies.

In the simplest case a correlation (or covariation) spectrum to a given reference is a very powerful correlation study. This could for example be the correlation between VIS and NIR spectra of pectin and the degree of esterification (%DE) (see Figure 1E.2) [4].

Figure 1E.2 highlights the impact of correlation studies for finding important regions in complex spectra without an actual model or if simple models are introduced. This simple case both illustrates the usefulness of the approach and the danger of misinterpretations. It is well known that the methyl ester stretch is found at 2244 nm in the near infrared spectrum (circle) and indeed a high positive correlation is found at this wavelength. However, the negative correlations found at
1990 nm and 2202 nm are stronger which basically is a simple case of the biological cage of covariance: when one correlation goes up others go down i.e. when pectins are esterified the amount of galactoronic acid goes down. This approach can directly be extrapolated to correlation analysis between two blocks of data. Direct correlation analysis between blocks of data was first explored by Noda in the 2-dimensional correlation method between data from different vibrational spectroscopies [5]. The results of this approach is a number of features which are highly correlated in the two blocks and it has in particular been exploited in NMR metabolomics in order to correlate different NMR methods [6], to correlate NMR data to disease status to obtain latent biomarker identification [7] and to correlate NMR data to UPLC-MS data in toxicology studies [8].

Such simple correlation analyses even before introducing any chemometric model are extremely powerful. Correlation analyses prior to subsequent data modelling can enhance data understanding, awareness of potential problems in data models, guide the choice of preprocessing method and emphasize incoherency in data obtained from different analytical platforms.

Correlations studies can be seen as a simple first step of the chemometric multiblock approaches mentioned at the end of this Example.

3 Multivariate challenges and opportunities

In classical empirical research a model requires that the number of variables must be less than the number of observations, but developments in modern analytical platforms have pushed scientists far beyond the classical model. In typical foodomics studies it is normal to include 100-500 samples. If these samples were to be measured by near infrared spectroscopy, such as in a classical QC analysis set-up, typically 1000 spectral variables are recorded. Such data sets of the order of 100 x 1000 can with advantage be dealt with using chemometrics which efficiently can model collinear data sets with many more variables than samples. However, nowadays it is not uncommon that the analytical platform record more than 100,000 variables for each sample which pushes the chemometric tools to the limit as this increase will also increase the chances of spurious correlations and include more interferences. Several techniques and strategies are available, not only for reducing the number of variables before the actual model, but also to reduce the number while modelling. When measuring many variables a large proportion of them will be irrelevant and it is advantageous to get rid of such irrelevant variables before data analysis. In foodomics studies irrelevant variables often means unsystematic variables, noisy variables or variables containing different information than the one explored (e.g. the difference between two diets). As variables in foodomics studies are often directly linked to metabolites, the term metabolites will also be used in the following.
The study of correlations between information found in single or multiple metabolites from multiple analytical platforms provides a novel way of validating the extracted information. A metabolic fingerprint would ideally represent the detection and quantification of all small molecules present in a biological system at a given time. However, the analytical platforms used for separation and detection of metabolites do not enable the identification of all in vivo metabolites, mainly owing to inadequate compound extraction and the dynamic range of the metabolome. Another issue is that different analytical platforms might not respond identical to the same metabolite [9]. The Venn diagrams, which show shared and unique metabolites from different analytical platforms, do not however, take into consideration the quantitative nature of the shared metabolites. Quantitative discrepancy might lead to too many reported metabolites being important and therefore a study of correlations between analytical platforms can provide valuable information with regard to removal of irrelevant metabolites.

The access to several analytical platforms in many foodomics labs makes the classical approach of using only one analytical platform - e.g. NMR or LC-MS [10-12] - suboptimal. The need for a comprehensive coverage of the foodome in untargeted studies calls for the use of complementary analytical platforms [13-18]. The use of more than one analytical platform will provide several sources of information as indicated in Table 1E.1.

Table 1E.1. Possible correlation studies with data from more than one analytical platform. A) Principal Component Analysis (PCA) on augmented data blocks B) Univariate regression, C) Multivariate regression (e.g. Partial Least Squares regression - PLS), D) Multiway regression (e.g. N-PLS) [19], E) Anova PLS [20], F) PLS2 regression, G) Multiway data blocks are normally transformed to be able to use case A-F. Not all cases will be illustrated in this Example - for more elaborated examples refer to the references given.
Very often the structure(s) of the data used for chemometric models depends on the routines applied in the foodomics lab. The data structures presented in the table can also be analyzed with more complex correlations studies (see references included in the different sections) which might be too involved to use in a standard foodomics setup. However, in the advanced set-up it is sometimes awarding to investigate correlations not only within blocks of data but also between blocks of data. How to approach multiway structure of e.g. LC-MS data in Foodomics studies will not be dealt with here but has been discussed in [21].

In order to highlight the advantage of multivariate correlation studies one can compare simple univariate and multivariate relationships on simple data as visualized in Figure 1E.3.

Univariate correlation studies are often too simple and often rather simple multivariate approaches of well selected variables can reveal much more of the underlying information in the data. Increasing the number of variables also increases the chance of finding even more (co)relations describing a similar difference between samples and as such confirms the findings presented in Figure 1E.3.
One area within foodomics where such observations can be particular useful is in the validation of extracted metabolites from CE-MS, GC-MS or LC-MS studies. This is normally performed using dedicated software packages that use different principles for extracting relative concentrations of the metabolites [22, 23]. Examples of software packages for high resolution mass spectrometry data are MZMine [24, 25], XC-MS [26, 27] and MetAlign [28, 29] (all freeware), vendor software such as Markerlynx (Waters), Sieve (Thermo) or ProfileAnalysis (Bruker), but also advanced peak deconvolution methods can be applied [21]. Which software to use is highly dependent on the research environment and the chosen analytical platform. The main issue is that they do not provide the same results - neither qualitatively (the found metabolites identified with a retention time m/z pair) nor quantitatively (the relative concentration found in the different samples) [30]. In a recent metabolomics study several software packages were compared and the shared and unique metabolites were reported [31]. Adding correlations studies on top of such data will further validate the shared metabolites and how the different software are capable of extracting the same information as visualized in Figure 1E.4.

In the study by Gurdeniz et al. the metabolites were not described in the same way by all software, which could however not be seen in the reported Venn Diagrams. This was only observed through subsequent (quantitative) correlation studies. Selecting just one software package will thus bias the results for some metabolites (blue boxes) whereas for other metabolites the outcome will be the same (red boxes).

This simple comparison demonstrates the need for correlations studies even within the same analytical platform; in fact quantitative consensus between software will most likely lead to even better foodomics studies. In a similar manner correlation studies will provide an additional validation in foodomics studies where the extraction of metabolites can be done in different ways.

In general the inspection of loading plots (as done in Figure 1E.4) is essential in correlation studies as loadings present all metabolite correlations consistently found in data. Depending on location of metabolites in the loading plots it is possible to extract valuable information pertaining to metabolite correlations and any blocks that can be combined can be evaluated. One of the difficulties though with loading plots is the pre-hand use of scaling of the data. Very often the visual appearance of the loadings plot will reflect the chosen scaling technique (e.g. no scaling, unit variance scaling, pareto scaling) which has the potential to deteriorate the real knowledge gain from this plot. As an example unit variance scaling on data with thousands of metabolites will provide a loading plot with a crowded centered circle with way too many metabolites being regarded as important (Figure
1E.5). How to evaluate such a loading plot is a tricky exercise. One alternative is to reduce the importance of low-intense metabolites by e.g. pareto scaling which has the advantage that less metabolites need to be examined, but also the risk of overlooking important low-intense metabolites.

One approach to circumvent running into too few/too many metabolites is to use the loadings plots in a combined manner. For example it can be recommended to use an approach where all three (or others or more) scaling techniques are evaluated simultaneously or to use so-called correlation loadings [32]. The latter is an approach in which a certain distribution of samples along the different principal components in e.g. a PCA score plot can be correlated to all metabolites (scores are correlated to metabolite intensities across samples). In this manner a distribution of samples found in an intervention study (as given in the score plot) can be investigated in all metabolite profiles regardless of intensity level in the raw data. This ensures that also low intense metabolite are evaluated in a given model space.

**Improved pre-processing through intra-block and inter-block correlation studies**

Data pretreatment, scaling and normalization are often essential for a successful foodomics study. Recorded metabolite profiles, measured from food or biofluid samples, are often subject to systematic variations in intensity across all measured variables which can mask the biologically relevant differences between profiles. The sources of obscuring variation may arise from inhomogeneity of samples, minor differences in sample preparation, instrumental perturbation and also data extraction steps may introduce an additional error [33]. Data normalization (vertical scaling between samples) and data scaling (horizontal scaling between variables) is typically applied to remove unwanted systematic bias in ion- or signal- intensity measurements while retaining the interesting biological information. While the normalization of metabolite profiles is intended to remove this spurious between-sample variation from the data, paradoxically, the application of any normalization procedure can also lead to deterioration of the biological information and the introduction of spurious correlations. The chosen normalization method can thus have a decisive effect on the outcome of subsequent application of pattern-recognition methods. There exist several established methods for normalizing metabolite profiles and each method is based on certain assumptions to the nature of the data. Some of the most common normalizations methods are: (1) unit sample intensity sum; (2) unit sample vector length (Euclidian norm); (3) probabilistic quotient/median fold-change [34]; (4) a reference feature present in all samples at a constant level; (5) histogram matching [35] which seek to match the histogram of
intensities in a spectrum as closely as possible to that of a reference spectrum; and (6) entropy related methods [21].

Since any normalization method may be destructive to the quantitative information buried in the metabolomics data, it is always recommended to use an external validation of the normalization method used. This can be optimally done if, for example, external quantitative knowledge from chemical reference parameters is available. Then, a simple multivariate regression to a given metabolite can evaluate if the normalization deteriorates or improves the correlation.

The use of external validation is demonstrated in a simple case study described below and shown in Figure 1E.6. The figure concerns a NMR study of human urine as a part of an intervention study concerning protein intake [36]. Urine samples have an inherent problem with normalization as the urine may contain different amounts of dry matter related to the study subject hydration status and time since last urination. Any measurement of urine thus requires normalization. In this case study both NMR spectra and chemical reference measurement of creatinine was measured for all urine samples. It was assumed that the best normalization method can then be selected as the one which conserve the creatinine information best [37] and this was studied through correlations analysis in form of PLS models.

Figure 1E.6 shows how the regression performance between the NMR spectra and the externally measured creatinine concentration/excretion can be influenced by different normalization methods. The normalization providing the best model (here mean centering and TSP normalization) will then serve as a good choice for how to pre-process the NMR data. If QC samples have been available the best pre-processing method could also have been studied in simple PCA models using proximity of the QC samples in the score plot as an optimization criterion. Other scaling and normalization methods have been suggested for foodomics data and in combination they serve as an important pre-step [38]. A limitation of this ‘supervised guidance’ of preprocessing methods can also be applied for other foodomics data blocks where external knowledge is available.

Together with the single block scaling and normalization issues mentioned above additional considerations are required when combing several blocks of data. When combining different blocks of data, scaling of the individual blocks becomes important. Often, there is orders of magnitude difference between the variations represented in the different blocks which will in turn bias the unsealed modelling. For example, one chemical compound may be represented in an NMR or LC-MS peak with one hundred data points while another metabolite may be represented in one or a few
variable(s). Such mismatch in magnitude of variation will lead to biased models, especially as most multivariate models favor high variation. Combining blocks of different nature (continuous, discrete, univariate etc.) is a challenging task and several methods have been proposed for achieving this [39-42]. Data blocks that originate from multiple analytical platforms will have very different ranges and noise characteristics. Scaling must be done carefully and, if possible, separately for each data block. However, not only scaling within each block is important, also the scaling between blocks is important because larger blocks become more dominant [43, 44].

The simplest solution to the multiblock scaling problem is to convert all blocks of data into discrete variables which can then be concatenated and scaled to unit variance (UV scaling). This ensures that all variables have an equal chance of affecting the model and that local and minor types of information/variation are easier found due to the noise reduction when turning continuous data into discrete variables. It is, however, important to remember that one block of data with many correlated discrete variables could still dominate the variation explained by the model and as such UV scaling does not ensure each block to be equally emphasized by the model. Besides UV scaling, other scaling techniques have been applied depending on the aim of the study, noise characteristics and block structure such as scaling to unit block variance [41] or other variance measures [16, 38, 45, 46].

When data blocks are augmented it is recommended to use metabolic profiling i.e. to turn all data blocks into discrete data. For NMR data this implies integrating and extracting peak areas and for LC-MS data this will normally imply to use software that generates peak tables consisting of metabolites with a unique retention time and mass pair [22, 23]. A PCA model with UV scaling will often be the first method of choice indicating similarities and differences between variables within and between blocks. If the blocks remain too different in complexity and co-variation it is recommended to investigate different block scaling methods as mentioned above. However, it should be emphasized that the final foodomics result will be biased depending of the chosen technique and as such this approach might not be trivial. This is why meta-data from the multivariate data modelling always must be reported in scientific literature.

**Simple methods - powerful correlations!**

For correlations analyses there are basically two ways to proceed. The simplest is to examine univariate correlations between design parameters to different metabolites, but this is rarely appropriate due to the multicomponent nature of most Foodomics studies. The other approach is to use multivariate methods for correlating many features at the same time thereby allowing
interactions to take part in the found correlations. For the latter part several correlations methods have been proposed in the literature such as matrix correlations, procrustes analysis [46], PLS2 and multiblock methods (see section 4).

How to check complimentary information in different analytical platforms?

To be able to validate information from one analytical platform with information from another platform is an efficient way of proving the validity of the experimental setup and the results [8]. With multiple analytical platforms such as NMR and LC-MS all combinations shown in Table 1E.1 can be investigated depending on how the raw data is treated (not taking cleaning, initial variable selection and preprocessing into consideration).

The simplest two-block case is scatter plots where (quantitative) correlations between metabolites can be visualized. The two vector scatter plot is the backbone of science and can be extremely powerful when linking metabolites from different data blocks, e.g. glucose found from NMR plotted against glucose found from LC-MS. However, in metabolomics studies the correlations are often not univariate but rather a combination of several metabolites acting together (a pattern) in response to a certain perturbation (e.g. different diets). These multivariate patterns or correlations can be visualized through latent variable methods such as PCA or PLS where the dimensionality of the block(s) can change from multivariate in NMR studies to multiway in LC-MS or GC-MS studies. Studying such correlations can be used to highlight and validate if the performance of the two analytical platforms are similar and if they measure the shared metabolites in the same way. One reason for this not to happen could be that the analytical platforms are not expected to extract a shared metabolite in the same way. For example if one method is less sensitive, this platform (e.g. NMR) might not capture the same trend as observed in a high resolution and sensitive platform such as LC-MS. Contrary if the concentration is too high it might affect the correlation due to detector issues such as ion suppression and saturation in MS.

Running the same samples on the same analytical platform (LC-MS), but in two different setups (polarity, column type etc.) can also enhance the understanding and validity of the acquired data. An example could be Hydrophilic Interaction (HILIC) and Reversed Phase (RPC) Liquid Chromatography data measured on the same samples [47]. For such data a preliminary PCA model on augmented data, a PLS2 model linking the two blocks or correlations based methods will indicate if the known similar information (both qualitative and quantitatively) can be found in both
analytical platforms. If this can be validated, complementary information (not expected to arise from both methods) will most likely also be valid and can then be further processed.

4 Multi-block methods and their potential in Foodomics - a brief look into the advanced possibilities with correlation studies

Above is discussed a number of simple and rather well established methods for relating data to each other. Focus was on univariate methods for relating two variables to each other and on multivariate interpretation of one data block/set and the relation between two data blocks. Below is a brief overview of some natural and quite recently developed extensions of these methods and also indicate how they can be potentially relevant in the present foodomics context (see [46, 48]). These methods are known under different names, the most used being multi-block methods, multi-set methods and data fusion methods.

The area of multi-block methodology [49, 50] is a very natural extension of methodologies such as PLS regression mentioned above, but instead of looking at the relation between two blocks of variables, multi-block methods can be used for relating several data blocks to each either for prediction or for interpretation purposes. The development has been fuelled by an increasingly strong need in several modern scientific disciplines with the -omics area as one of the most important. Some of the methodologies developed fit perfectly into the area of Foodomics not only because of the data structures available but also because of the needs for interpreting complex relations between several large data sets (NMR, XC-MS, GC/LC etc.). The concepts involved are strongly related to standard multivariate analysis (see e.g. [51]) which implies that very similar ways for visualizing and interpreting the results can be used.

The area of multi-block modelling can broadly be split in two closely related disciplines based on the purpose of the analysis. 1) If predicting and understanding how a number of different blocks can be combined for predicting one or several other blocks, the area is called multi-blocks regression (and is a direct extension of PLS2 regression as discussed above). A typical example of this is when several blocks are used for gaining information about for instance a number of chemical constituents or about which groups of samples a specific sample belongs to [42]; 2) In other cases no specific order of the blocks is of interest, only how the blocks are related [46].

The most natural way of approaching the two situations is by the use of so-called data-compression methods inspired by the original PCA. These are methods that are based on stable projections of the data and visual inspection and empirical validation of the extracted components.
These are methods which are developed for solving the collinearity problem (i.e. many more highly correlated variables than the number of samples) and a number of efficient calculation and estimation methods already exist [52].

In multi-block modelling, there are a number of concepts that have appeared to be very fruitful and that are also relevant in Foodomics studies. Among these concepts are common variability, unique variability and additional variability of the blocks (see e.g. [42]), the latter mainly relevant for regression situations. The former of these has to do with the dimensions defining the joint variability that is common in the blocks [53], i.e. the linear combinations in one of the blocks that are highly correlated with linear combinations in the other. One can also think of this as the intersection between the spaces defined by the by the blocks. The unique dimensions of the blocks are those directions in the block spaces that are uncorrelated with the common variability, in this sense representing information that is not shared by the other blocks. Additional information is related to prediction and defines how and how much extra information each of the blocks contributes to the prediction of output variables. All these concepts are quite general and can be implemented in various ways. Their future within Foodomics is quite obvious. For instance, one is often interested in what is common and what is unique in different information sources (as was also touched upon in the correlation studies above) and sometimes the methodologies are used for classification which clearly falls under the prediction concept above. Some of the operationalizations of the concepts are also invariant (see e.g. [42]) to the relative scaling of the blocks which can make them particularly important when data blocks of different type are involved in one single analysis.

5 Outreach

Foodomics studies are complex and studies dealing with foodomics data are concerned with multifactorial problems, and as these are analyzed with multifactorial sensors and separation methods multivariate data handling methods are required to extract and describe the data. The modern analytical platforms generate vast amount of data in a very short time and the analyst risks the challenge to be flooded with non-informative data. There are multiple ways to analyze foodomics data and many choices have to be made. Data recording, cleaning, preprocessing, modelling and validation are all essential parts of the informatics flow in any foodomics study. However, multivariate data analysis typically remains the bottleneck for most systems biology studies, including foodomics, and new developments in chemometrics are required for the challenging data fusion or meta-analysis often demanded in system biology studies. Obligatory
Deposit of large foodomics data sets published in the literature should serve as an inspiration for innovative chemometricians.

However, simple correlations studies (with well known chemometric principles from the foodomics world) have proven to be very powerful methods for a first glance of the relationship between multiple blocks of data. Such studies could be a part of the generic data handling chain in all future foodomics studies.

Finally, it cannot be stated too strongly that any result from a properly designed foodomics study will rely on the quality of the internal and external validation and last but not least that it will ultimately be necessary to validate the found correlations with evidence from other sources because statistical methods cannot discriminate between causal effects and indirect correlations.

6 References for Example 1E


Example 1F - Development of a chemically defined medium for studying foodborne bacterial-fungal interactions

There is a growing interest for the use of natural preservatives in the food and dairy industry including the application of bacterial cultures to inhibit spoilage by molds and yeast. Several antifungal metabolites from bacteria have been identified, but their relative importance has been difficult to establish. In dynamic systems such as fermented milk products which display spatio-temporal changes in acidity and texture, the complexity of the food matrix affects detection, identification and quantification of antifungal metabolites and thereby impedes the understanding of the bacterial-fungal interactions.

In order to ease the identification and relative quantification of metabolites produced by bacterial cultures (as judged by Ultra Performance Liquid Chromatography/mass spectrometry) a chemically defined interaction medium (CDIM) was developed. The medium supported growth of cultures associated with antifungal activity such as Lactobacillus paracasei and Propionibacterium freudenreichii subsp. shermanii as well as spoilage molds and yeast (Penicillium spp., Rhodotorula mucilaginosa and Debaryomyces hansenii) commonly found in fermented milk products. Both strong and weak antifungal interactions observed in milk could be reproduced in CDIM. The medium thus seems suitable for studying antifungal activity of dairy bacterial cultures. It further enables the use of optical methods for studying microbial growth or inhibition, and enables modification or labelling of compounds which will facilitate the study of metabolites and antifungal mechanisms.

Abbreviations

CDIM Chemically defined interaction medium
CDIM-C- Ferment of low inhibitory strains of Lb. paracasei in CDIM
CDIM-C+ Ferment of Lb. paracasei DGCC 2.132 with cells in CDIM
CDIM-CF+ Cell free ferment of Lb. paracasei DGCC 2.132 in CDIM
LAB Lactic acid bacteria
MEB Malt extract broth
MEA Malt extract agar
MIC Minimum inhibitory concentrations
Milk-C+ Ferment of Lb. paracasei DGCC 2.132 with cells in milk
Milk-CF+ Cell free ferment of Lb. paracasei DGCC 2.132 in milk
MRS Man Rogosa Sharpe
MS Mass spectrometer
m/z Mass to charge ratio
PAB Propionic acid bacteria
QTOF Quadrupole time-of-flight
SLB Sodium lactate broth
SLA Sodium lactate agar
UPLC Ultra Performance Liquid Chromatography

1. Introduction

Spoilage of food by fungi is causing major losses in the food industry every year. The use of bacterial cultures to control fungal growth has gained increased interest in the food industry due to consumers’ demands for reduced use of chemical preservatives. Several studies have investigated aspects of the complex nature of the antifungal activities displayed by several lactic acid bacteria (LAB), with or without propionic acid bacteria (PAB) [1-3]. In several studies, some extracellular metabolites have been identified by fractionating supernatants of bacterial ferments followed by antifungal activity test of individual metabolites (Sjogren et al., 2003; Yang & Chang, 2010). When single compounds have been tested, the minimum inhibitory concentrations (MIC) have typically been in the order of 100-1000 fold higher than the reported levels produced by the bacteria (Ryan, Dal Bello, Arendt, & Koehler, 2009; Schwenninger et al., 2008). It has therefore been suggested that synergistic effects between antifungal metabolites must be responsible for the inhibitory activity (Magnusson & Schnurer, 2001; Magnusson, Strom, Roos, Sjogren, & SchnGrer, 2003) but there is still limited understanding of the mechanisms and significance of the different metabolites produced.

The identification of all antifungal metabolites produced during fermentation poses a major challenge due to the complexity and variation of many dairy matrices. Milk is in itself a complex food medium being an aqueous solution of lactose, organic and inorganic salts, vitamins, and other small molecules, in which proteins and lipids are dispersed and where the majority of the proteins, namely caseins, exist as colloidal aggregates (Fox & McSweeney, 1998a, 1998b). The metabolism of starter cultures induces further matrix complexity since proteolytic activity of LAB causes hydrolysis of milk proteins into peptides (Savijoki, Ingmer, & Varmanen, 2006) and acidification to pH ca. 4.6 destabilizes the casein micelles resulting in gelling and precipitation (Fox, 2009). The search for secreted microbial metabolites therefore becomes hampered by the presence of a large variation of peptides from specific and non-specific enzymatic hydrolysis of milk proteins (Lawa & Haandrikmat, 1997) and by several sample preparation steps.
A chemically defined medium can be used as a reproducible model system to simplify the initial detection of metabolites by reducing the background noise and simplifying the sample preparation (Brosnan, Coffey, Arendt, & Furey, 2012). With fewer preparation steps the probability of identifying and following metabolic dynamics at the molecular level increases. Furthermore, a chemically defined medium can be modified in order to investigate the role of specific nutrients or precursors of antifungal compounds. Chemically defined media have usually been optimized to a specific strain or sub-species while studies on multi-species interaction in defined medium are limited.

Here, is reported the development of a chemically defined interaction medium (CDIM) which supports growth of relevant LAB, PAB, molds and yeast and, in which, the antifungal activity of LAB and PAB in CDIM was comparable to that observed in a milk based medium. Apart from the greatly improved possibility for differentiating and identifying metabolites, the transparent nature of CDIM also allows for direct monitoring of growth of LAB as well as target organisms by optical methods.

2. Materials and Methods

2.1 Chemicals and solvents

All listed chemicals and solvents were from Sigma-Aldrich (Schnelldorf, Germany) and all water was freshly produced Milli-Q quality (Merck Millipore, Billerica, MA, USA) unless otherwise specified. 2-hydroxy-3-phenylpropanoic acid (=‘3-phenyllactic acid’) and 2-hydroxy-3-(4-hydroxyphenyl)propanoic acid (=‘3-(4-hydroxyphenyl)lactic acid’) were purchased from Sigma.

2.2 Microbial strains and media

The microbial strains and their growth conditions are listed in table 1F.1. The bacteria and fungi used in these studies were supplied by DuPont Nutrition Biosciences ApS. All strains were kept in their growth medium with 20% glycerol at -80 °C until use, except for mold spores which were stored in water with 0.01% tween80. Indicator fungi were originally isolated from spoiled fermented milk products. All molds and yeast strains were able to grow in yogurt.

2.3. Preparation of chemically defined interaction medium

A CDIM was developed on the basis of defined media previously reported for growth of LAB (Morishita, Fukada, Shirotta, & Yura, 1974; M0retr0, Hagen, & Axelsson, 1998; Saguir & de Nadra, 2007; Savijoki, Suokko, Palva, & Varmanen, 2006), PAB (Dherbecourt, Maillard, Catheline, &
Thierry, 2008; Glatz & Anderson, 1988), molds (Bockelmann, Portius, Lick, & Heller, 1999; Emeh & Marth, 1976; Meyers & Knight, 1958) and yeast (Andersen, Renshaw, & Wiebe, 2003; Hobot & Jennings, 1981) respectively. The medium was combined based on minimum requirements of any of the species investigated. CDIM was prepared from concentrated stock solutions of chemicals in water (table 1F.2). The pH was adjusted to 6.5 and the medium filter sterilized through a sterile 0.2 μm pore size filter (Nalgene®) prior to use.

CDIM, acidified with DL-lactic acid to pH 4.5 corresponding to the pH level of CDIM fermented with bacterial cultures, was used as a reference in order to investigate the effect of acidification alone. Plates of CDIM were prepared by mixing with agar to a final concentration of 1% agar.

2.4. Growth in CDIM vs. standard media

Bacterial growth: Growth of Lb. paracasei DGCC 2132 (10^7 cfu mL⁻¹) was measured by following OD₆₅₀ every second hour during incubation in sterile flat-bottomed 96 well microplates (Fisher Scientific). Microplates were incubated at 37 °C for 40 hours using a Varioskan Flash (Thermo Fisher Scientific Oy, Finland). Growth of P. freudenreichii subsp. shermanii DGCC 2053 (10^7 cfu mL⁻¹) requires anaerobic conditions and was therefore measured by cfu mL⁻¹ after 4 days of anaerobic incubation at 30 °C. The standard media MRS and SLB were used to compare growth of Lb. paracasei DGCC 2132 and P. freudenreichii subsp. shermanii DGCC 2053, respectively.

Molds: Mold growth was tested on plates of CDIM at both pH 6.5 and 4.5 (acidified with DL-lactic acid) and tested by spotting 20 μL of 10^5 spores mL⁻¹ (2x10^3 spores) of each Penicillium strain. Plates were incubated at 25 °C for 3-5 days until visible growth. Growth of molds on MEA was used for comparison.

Yeast: Liquid CDIM (pH 6.5) was inoculated with 10⁵ cfu mL⁻¹ of D. hansenii DCS 605 and R. mucilaginosa DCS 1063, respectively, and incubated at 25 °C for 4 days. Growth was measured by OD₆₅₀ as previously described. Yeast growth on plates of CDIM was tested by spotting 10 μL of the yeast concentrations 10³, 10⁴ and 10⁵ cfu mL⁻¹. Growth of yeast on MEA was used for comparison.

All determinations were done in triplicate.

2.5. Antifungal activity tests in CDIM compared to milk
Co-cultures of LAB and PAB in the ratio 20:1 were prepared by inoculating CDIM and UHT milk, respectively with the antifungal *Lb. paracasei* DGCC 2132 together with *P. freudenreichii* subsp. *shermanii* DGCC 2053. Batches were fermented at 37 °C for 22 hours to obtain ferment with cells (C) of the inhibitory strain (+) from milk (milk-C+) and CDIM (CDIM-C+) respectively. CFU mL⁻¹ of *Lb. paracasei* DGCC 2132 and *P. freudenreichii* subsp. *shermanii* DGCC 2053 were determined after end of fermentation. Similarly, co-cultures of low inhibitory strains (-) of *Lb. paracasei* (DGCC 693 and 477) and *P. freudenreichii* subsp. *shermanii* DGCC 2053 were prepared in CDIM to obtain a cell containing ferment with expected low antifungal activity (CDIM-C-) (table 1F.3). Plates of ferment were prepared by mixing with agar (1%). Plates of CDIM and milk, respectively, without added bacteria were prepared as reference.

Fungal inhibition: Plates of CDIM-C+, milk-C+ and reference plates of CDIM and milk were spotted with 20 μL of a 10⁵ spores mL⁻¹ solution of *P. solitum* DCS 302 and *Penicillium* spp. DCS 1541. CDIM-C- were spotted with *P. solitum* DCS 302. Plates of CDIM-C+ were spotted with 10 μL of yeast cell concentrations of 10³, 10⁴ and 10⁵ cfu mL⁻¹ in triplicates.

All plates with spotted molds and yeast were incubated at 25 °C for 3-5 days. CDIM without added bacteria were used as reference.

2.6. Chemical profile of cell free ferments from chemically defined medium and milk

Cell free ferments of *Lb. paracasei* DGCC 2132 (CF+) was prepared to study the chemical profile of CDIM and milk respectively. CDIM-C+ and milk-C+ was placed on ice water immediately after fermentation and centrifuged at 4500 x g for 10 minutes at 0 °C. The supernatants were filtrated through a sterile 0.45 μm pore size filter (cellulose acetate membrane, Q-Max, Frisenette Aps) to obtain cell free ferments of CDIM (CDIM-CF+) and milk (milk-CF+) respectively. CF+ (200 μL) was mixed with 0.1% v/v formic acid (800 μL) in water containing 0.06 mmol/L L-phenyl-D₅-alanine and 0.08 mmol/L 1,2-¹³C₂-L-leucine. The diluted sample solution was centrifuged at 13300 x g for 10 minutes prior to analysis. Verification of compound identity was performed by analyzing CF spiked with 0.1 mg/ml of the reference chemical.

Samples were analyzed using an Agilent (Agilent Technologies, Waldbronn, Germany) modular 1290 Ultra Performance Liquid Chromatography (UPLC) instrument coupled to a Bruker (Bruker Daltonics, Billerica, MA) maXis 4G single quadrupole time-of-flight (QTOF) mass spectrometer (MS) via an electrospray interface. The UPLC was mounted with a Waters (Waters Corporation, Milford, Ma, USA) HSS T3, 2.1 x 150 mm ID column packed with 1.8 μm particles. Mobile phases were A)
water/formic acid 1000/1 v/v and B) acetonitrile/formic acid 1000/1 v/v. Vials were kept at 5 °C in the auto sampler prior to injection of 10 μL. Elution was performed with a flow of 400 pL/min and a gradient starting at 0% B at t=0 and kept for 1.0 min, then 100% B at 15 min for 0.5 min, and back to 0% B over 0.1 minutes and maintained for 4.4 min. The electrospray interface with nebulizer at 2.5 bar and dry gas at 9.0 L/min at 200 °C was operated in both positive and negative mode (capillary voltage at 4000 V and 3200 V). Mass spectra in the range mass to charge ratio (m/z) 60 - 1250 were acquired with a frequency of 3 Hz. The instrument was controlled using Bruker Daltonics micrOTOF control version 3.1 and acquired data was handled with DataAnalysis version 4.0 SP4.

3. Results and Discussion

3.1. Growth in CDIM medium

A chemically defined growth medium for interaction studies was developed based on growth requirements of dairy relevant LAB and PAB, yeast, and molds. The growth behavior in CDIM was compared with growth in the respective standard media for each type of microorganism (MRS, SLA or MEA). The transparency of CDIM allowed for use of optical methods for assessing growth. All microorganisms tested were able to grow in CDIM. Compared with growth on their standard rich media, either somewhat lower growth rates and final levels as in the case of the bacteria (fig 1F.1), slightly higher growth rates as seen for the yeast (fig 1F.2) or same growth as seen in the case of molds (fig 1F.3) was observed. *Lb. paracasei* DGCC 2132 had a generation time of 4.7 and 1.9 hours in CDIM and MRS, respectively. Lower growth rates in chemically defined medium compared to rich medium is well known (Jensen & Hammer, 1993). These differences in cell growth might be related to better accessibility of nutrients, e.g. peptides in the complex MRS medium, compared to amino acids in chemically defined medium (Van Niel & Hahn-Hagerdal, 1999). Decreased growth of bacteria in CDIM could be an indication of stress. Since stress is known to influence the metabolism of LAB (Van de Guchte et al., 2002), this should be considered when analyzing the produced metabolites.

Both *D. hansenii* DCS 605 and *R. mucilaginosa* DCS 1063 grew in liquid CDIM and MEB (fig. 1F.2) reaching stationary phase after 4 days. Growth of yeast on plates of solid CDIM and MEA was observed after 3 days of incubation (fig. 1F.2). *P. freudenreichii* subsp. *shermanii* DGCC 2053 is known to be slow-growing (Falentin et al., 2010) and increased with a little less than 2 and 2.5 log (cfu mL⁻¹) in CDIM and SLB, respectively, after 4 days of anaerobic incubation at 30 °C.
On plates of both solid CDIM and MEA growth of *P. solitum* DCS 302 and *Penicillium* DCS 1541 was observed after 2 days and sporulation appeared after 3 days, with heavy sporulation on day 4 (fig. 1F.3).

In addition to the mentioned organisms, 9 strains of *Lb. casei*, 1 strain of *Lactobacillus rhamnosus*, 2 strains *Penicillium* spp. and 2 strains of *Fusarium* spp. were tested and grew well in CDIM (data not shown).

### 3.2. Antifungal activity

CDIM-C+ inhibited growth of all indicator fungi tested with molds being more sensitive than yeast. Growth of *Lb. paracasei* DGCC 2132 in milk and CDIM was similar after 22 hours of fermentation reaching 8.03±0.04 and 8.32±0.07 log(cfu mL⁻¹) in CDIM-C+ and milk-C, respectively. The pH, however, dropped to a lower level in CDIM-C+ (from 6.5 to 4.4) than in milk (from 6.6 to 5.1). No growth of *P. freudenreichii* subsp. *shermanii* DGCC 2053 was observed in either CDIM or milk.

Molds spotted on plates of CDIM-C+ and milk-C+ were inhibited for 4 days (fig. 1F.4).

Growth and sporulation appeared on CDIM-C+ after prolonged incubation, whereas growth but no sporulation was observed on milk-C+ (data not shown). The trace element copper is known to support sporulation of molds (Samson, Houbraken, Thrane, Frisvad, & Andersen, 2010) and different vitamins belonging to the B-group, especially thiamine (Basu & Bhattacharyya, 1962) have also been shown to promote sporulation of *Penicillium* species. Both copper and B vitamin content except for riboflavin and Ca pantothenate (Saxholt et al., 2008) is higher in CDIM than in milk, which may explain the increased sporulation of molds on CDIM-C+. Decreasing pH with lactic acid had little effect on growth of molds (data not shown). In the case of yeast, the degree of inhibitory activity of CDIM-C+ depended on the target yeast with *R. mucilaginosa* DCS 1063 being more sensitive than *D. hansenii* DCS 605.

The low antifungal activity of two strains of *Lb. paracasei* observed in milk compared with *Lb. paracasei* DGCC 2132 was confirmed in CDIM when tested in co-culture with *P. freudenreichii* subsp. *shermanii* DGCC 2053 (fig. 1F.5).

### 3.3. Comparison of the chemical profile of cell free ferments from UHT milk and CDIM

The chemical profile of milk-CF+ and CDIM-CF+ was compared in order to demonstrate the potential for detecting produced metabolites without any sample preparation except for dilution. By analyzing CF+ without prior sample preparation such as filtrations or extractions, any loss of
relevant metabolites was minimized. Previous studies for identification of antifungal compounds such as phenyllactic acid produced in MRS, have demonstrated that even a one-step solid phase extraction sample preparation caused a decrease in recovery to only 10% of spiked-in amounts (Armaforte, Carri, Ferri, & Caboni, 2006). Similar observations was made by Brosnan and coworkers (Brosnan et al., 2012).

Detection by mass spectrometry requires ionization of the analytes of interest in the electrospray interface coupling the chromatography system and the mass spectrometer. Depending on the nature of the analyte and given chromatographic conditions, the compound will preferably become positively or negatively ionized. Compounds with acidic protons, like carboxylic acids tend to form negative ions, e.g. [M-H]⁻, whereas bases, like amines tend to form cations, e.g. [M+H]⁺. In order to detect as many compounds as possible in the ferments, the column eluate was monitored both in positive and negative mode.

Bovine milk contains a complex mixture of several hundreds of proteins with the major part being caseins (Gagnaire, Jardin, Jan, & Lortal, 2009). When milk is subjected to LAB, caseins are partly hydrolyzed to oligomers with a length of 4-18 by a range of cell-envelope proteases (Savijoki, Ingmer, et al., 2006). The chemical profile of milk-CF+ displayed a high amount of compounds eluting in the range ca. 4.5 - 9 min in positive electrospray mode as shown in the total ion chromatograms (fig. 1F.6). The density plot showed that the main part of the ions in the region resided in the m/z region above 400. Inspection and charge state deconvolution of mass spectra revealed that a major part of the compounds eluting in the 4.5 range were multiple charged, with charge states being 3 - 13 corresponding to molecular masses ranging from 1 to 25 kDa, probably originating from peptides. The large diversity of chromatographic peaks and highly populated MS-spectra corresponded with the complexity expected from peptides from partial hydrolysis of a diverse medium like bovine milk (Gagnaire et al., 2009). Due to the excessive signal from the peptides throughout the chromatogram, it is likely that the intensity of each of the observed signals will be affected by co-eluting compounds, either as ion suppression or enhancement (Covey et al., 2010). Besides the effect on MS signals due to matrix dependent ionization, the high loading of milk matrix component like triglycerides and protein will deposit both in the column over time affecting the chromatographic system as well as the interface. Even the analysis of a limited number samples of Milk-CF+ resulted in severe build-up of matrix components in the interface and continuous reduction of signal intensities (data not shown). Further analytical work with Milk-CF+ would have required additional sample preparation steps such as filtration and/or precipitation of proteins in contrast to the analysis of CDIM-CF+.
CDIM-CF+ displayed a significantly reduced complexity compared with milk-CF+. The chromatogram contained a majority of singly charged components with m/z below 600 and a much decreased overlap of mass spectral and chromatographic peaks. Some of the previously reported antifungal compounds (Schwenninger et al., 2008), 3-phenyllactic acid and 3-(4-hydroxyphenyl)lactic acid may therefore be readily identified (fig. 1F.7) and separated from other new metabolites formed. The CDIM-CF+ was thus more suitable for direct UPLC/MS analysis than milk-CF+ and it furthermore allowed the use of optical density for growth studies. It would also enable the use of labeled compounds for future metabolic studies of mixed cultures. However, since milk components may interact with metabolites or compartmentalization takes place during milk acidification, it is necessary to do final validation of biological activity and chemical changes in the relevant food system.

4. Conclusion

CDIM fulfilled the basic requirements for growth and showed similar results in the microbial interaction studies as for milk. CDIM could hence be used as replacement for milk to simplify the initial identification of metabolites produced by bacteria during fermentation. Detection and quantification of relevant metabolites in more complex media, such as yoghurt, will then be possible after having performed the initial assignment of the metabolites of interest.

References for Example 1F


Brosnan, B., Coffey, A., Arendt, E. K., & Furey, A. (2012). Rapid identification, by use of the LTQ Orbitrap hybrid FT mass spectrometer, of antifungal compounds produced by lactic acid
bacteria. Analytical and bioanalytical chemistry, 403, 2983-2995. doi:10.1007/s00216-012-5955-1


Tables

Table 1F.1
Microbial strains evaluated in these studies

<table>
<thead>
<tr>
<th>Microbial strain</th>
<th>Growth medium</th>
<th>Incubation temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactobacillus paracasei DGCC 2132</td>
<td>MRS</td>
<td>37 °C</td>
</tr>
<tr>
<td>Lactobacillus paracasei DGCC 693</td>
<td>MRS</td>
<td>37 °C</td>
</tr>
<tr>
<td>Lactobacillus paracasei DGCC 477</td>
<td>MRS</td>
<td>37 °C</td>
</tr>
<tr>
<td>Propionibacterium freudenreichii subsp. shermanii DGCC 2053</td>
<td>SLB/SLA</td>
<td>30 °C</td>
</tr>
<tr>
<td>Penicillium spp. DCS 1541</td>
<td>MEA</td>
<td>25 °C</td>
</tr>
<tr>
<td>Penicillium solitum DCS 302</td>
<td>MEA</td>
<td>25 °C</td>
</tr>
<tr>
<td>Debaryomyces hansenii DCS 605</td>
<td>MEB/MEA</td>
<td>25 °C</td>
</tr>
<tr>
<td>Rhodotorula mucilaginosa DCS 1063</td>
<td>MEB/MEA</td>
<td>25 °C</td>
</tr>
</tbody>
</table>

MRS, Man Rogosa Sharpe (Oxoid)
SLB, sodium lactate broth (20 g/L peptone (Becton Dickinson), 10 g/L yeast extract (Oxoid), 16 g/L 50% sodium lactate solution (Merck))
SLA, sodium lactate agar (SLB with 15 g/L agar (Becton Dickinson))
MEB, malt extract broth (30 g/L malt extract (Becton Dickinson), 5 g/L peptone)
MEA, malt extract agar (MEB with 15 g/L agar)

Table 1F.2
Composition of chemically defined medium used for growth of Lb. paracasei (LAB), P. freudenreichii (PAB), Penicillium spp. (Mold), D. hansenii (Yeast) and R. mucilaginosa (Yeast). x indicates which compounds have been reported to support growth of the different microorganisms.

<table>
<thead>
<tr>
<th>Components</th>
<th>LAB</th>
<th>PAB</th>
<th>Mold</th>
<th>Yeast</th>
<th>CDIM</th>
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<tr>
<td>Glucose</td>
<td>x</td>
<td>x</td>
<td>x</td>
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<tr>
<td>K₂HP0₄</td>
<td></td>
<td></td>
<td>x</td>
<td>x</td>
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<td>x</td>
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<td>x</td>
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<td>(NH₄)₂SO₄</td>
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<td>L-tyrosine (^a)</td>
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<td>Thiamine hydrochloride</td>
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<td>Riboflavin</td>
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<tr>
<td>Calcium</td>
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<tr>
<td>Pyridoxal HCl</td>
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<tr>
<td>Folic acid (^c)</td>
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<tr>
<td>Cyanocobalamin</td>
<td>x x x 0.0001</td>
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<td></td>
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</tr>
</tbody>
</table>

\(^{a}\) Dissolved in HCl
\(^{b}\) Added directly to medium
\(^{c}\) Dissolved in NaOH

Table 1F.3
Overview of ferments of inhibitory and low inhibitory strains of *Lb. paracasei* (LAB) used for antifungal activity test and chemical analysis.

<table>
<thead>
<tr>
<th>Type of ferment</th>
<th>Description</th>
<th>Used for</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDIM-C+</td>
<td>Inhibitory LAB strain grown in CDIM (with cells)</td>
<td>Antifungal activity test</td>
</tr>
<tr>
<td>CDIM-C-</td>
<td>Low inhibitory LAB strains grown in CDIM (with cells)</td>
<td>Antifungal activity test</td>
</tr>
<tr>
<td>Milk-C+</td>
<td>Milk with cells of inhibitory LAB strain</td>
<td>Antifungal activity test</td>
</tr>
<tr>
<td>CDIM-CF+</td>
<td>Cell free ferment of inhibitory LAB strain grown in CDIM</td>
<td>Chemical profile analysis</td>
</tr>
<tr>
<td>Milk-CF+</td>
<td>Cell free ferment of inhibitory LAB strain grown in milk</td>
<td>Chemical profile analysis</td>
</tr>
</tbody>
</table>

Example 1G - Contribution of volatiles to the antifungal effect of *Lactobacillus paracasei* in defined medium and yogurt

Summary
- Volatiles are important in antifungal activity of *Lb. paracasei*
- Diacetyl was one of the main volatiles responsible for the anti-mold effect
- Added diacetyl was antifungal in solid medium and yogurt at relevant concentrations
- Cell removal caused loss of volatiles and antifungal activity

Lactic acid bacteria with antifungal properties can be used to control spoilage of food and feed. Previously, most of the identified metabolites have been isolated from cell free ferment of lactic acid bacteria with methods suboptimal for detecting possible contribution from volatiles to the antifungal activity. The role of volatile compounds in the antifungal activity of *Lactobacillus*
paracasei DGCC 2132 in a chemically defined interaction medium (CDIM) and yogurt was therefore investigated with a sampling technique minimizing volatile loss. Diacetyl was identified as the major volatile produced by Lb. paracasei DGCC 2132 in CDIM. When the strain was added to a yogurt medium diacetyl as well as other volatiles also increased but the metabolome was more complex. Removal of Lb. paracasei DGCC 2132 cells from CDIM ferment resulted in loss of both volatiles, including diacetyl, and the antifungal activity towards Penicillium. When adding diacetyl to CDIM or yogurt without Lb. paracasei DGCC 2132, marked inhibition was observed. Besides diacetyl, the antifungal properties of acetoin were examined, but no antifungal activity was observed. Overall, the results demonstrate the contribution of diacetyl in the antifungal effect of Lb. paracasei DGCC 2132 and indicate that the importance of volatiles may have been previously underestimated.

Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>AL</td>
<td>Alpha-acetolactate</td>
</tr>
<tr>
<td>ALDC</td>
<td>Alpha-acetolactate decarboxylase</td>
</tr>
<tr>
<td>ALS</td>
<td>Alpha-acetolactate synthase</td>
</tr>
<tr>
<td>AR</td>
<td>Acetoin reductase</td>
</tr>
<tr>
<td>CDIM</td>
<td>Chemically defined interaction medium</td>
</tr>
<tr>
<td>C-ferment</td>
<td>Cell containing ferment</td>
</tr>
<tr>
<td>CF-ferment</td>
<td>Cell free ferment</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>GUI</td>
<td>Graphical User Interface</td>
</tr>
<tr>
<td>LAB</td>
<td>Lactic acid bacteria</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimal inhibition concentration</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>REF</td>
<td>Reference, acidified non-inoculated CDIM</td>
</tr>
<tr>
<td>SHS</td>
<td>Static headspace analysis</td>
</tr>
<tr>
<td>SPME</td>
<td>Solid phase microextraction</td>
</tr>
<tr>
<td>UHT</td>
<td>Ultra-high-temperature</td>
</tr>
</tbody>
</table>

1. Introduction

Antifungal lactic acid bacteria (LAB) have been studied in a wide range of foods and feed like sour-dough (Black, Zannini, Curtis, & Ganzle, 2013; Lavermicocca et al., 2000; Ryan et al., 2011), dairy products (Schwenninger & Meile, 2004; Voulgari et al., 2010) fermented vegetables (Yang & Chang, 2010), and silage (Strom, Sjogren, Broberg, & Schnurer, 2002). While most of the efforts
have been directed toward finding new potent strains, there is an increasing interest in understanding the antifungal mechanism including the identification and quantification of bioactive compounds produced by these strains. Several recent reviews exist on antifungal compounds produced by LAB (S. Crowley, Mahony, & van Sinderen, 2013; Dalie, Deschamps, & Richard-Forget, 2010; Schnirrer & Magnusson, 2005; Schwenninger, Meile, & Lacroix, 2011). However, the reported amounts produced by antifungal LAB seems to be far lower than what is needed as minimum inhibitory concentration (MIC) (Ryan, Dal Bello, Arendt, & Koehler, 2009; Schwenninger et al., 2008).

Schwenninger and Meile (2004) described the antifungal properties of a co-culture of Lactobacillus paracasei subsp. paracasei and Propionibacterium jensenii in fermented milk and cheese. They concluded that the inhibition was not solely based on the organic acids produced since acetic and propionic acid did not fully explain the antifungal effect (Schwenninger et al., 2008). Furthermore, some observations indicate a loss of antifungal activity upon cell removal (Schwenninger & Meile, 2004). This could point towards part of the antifungal effect being due to compounds that need to be continuously supplied from the cells, and hence are either labile, volatile or being consumed.

Bacteria can produce a wide range of volatile organic compounds (Kai et al., 2009; Schulz & Dickschat, 2007). In spite of this, most of the studies on antifungal compounds focus on the non-volatile, liquid cell free ferment, often using bioassay-guided fractionation (Strom et al., 2002) and/or pre-concentration, e.g. extraction and drying (Schwenninger et al., 2008). These concentration techniques are well suited for concentrating compounds less volatile than water (or the solvent). However, compounds more volatile than water will be lost in the process. The aim of this Example was to examine the role of the major volatile compounds produced by the antifungal Lactobacillus paracasei DGCC 2132. Fermentation were screened in both chemically defined interaction medium (CDIM) and milk using qualitative and quantitative methods and minimal sample processing, in order to elucidate the volatile profile. The level of a major volatile compound identified, diacetyl, and the antifungal effect towards selected fungal spoilers was compared in cell containing as well as cell free ferments. The activity of cell-containing ferment was also investigated after diacetyl formation had been inhibited by converting the precursor enzymatically.

The antifungal effect of added diacetyl and acetoin was furthermore examined at concentrations comparable to those produced by Lb. paracasei DGCC 2132. The results help to explain the role of diacetyl in the antifungal effect of Lb. paracasei DGCC 2132 and highlight the likely importance of volatiles.
2. Materials and methods

2.1 Chemicals and materials
2,3-butanedione (diacetyl) with purity 97%; 3-hydroxy-2-butanone (acetoin) with purity $\geq 96%$; Hydrochloric acid (37%) and 2-hydroxypropionic acid (DL-lactic acid) $\geq 85$% were purchased from Sigma-Aldrich (Schnelldorf, Germany). Alpha-acetolactate decarboxylase, ALDC (3000 ADU/g) was supplied by DuPont Nutrition Biosciences Aps (Brabrand, Denmark). Tween80 was from Merck, UHT Milk was from MILSANI®

2.2 Microbial strains, Media and Growth Conditions

Lb. paracasei DGCC 2132, previously found to have high antifungal activity in milk based systems, was used in these studies. YO-MIX™ 410 starter culture (DuPont Nutrition Biosciences Aps, Denmark) was used for production of yogurt. Freeze dried bacteria were stored at -18 °C until use. Penicillium solitum DCS 302 and Penicillium sp. DCS 1541 isolated from fermented milk products were used as indicator molds. Molds were grown on malt extract agar (Galloway and Burgess, 1952) (MEA, 30 g/L malt extract, 5 g/L peptone, 15 g/L agar) for 5-7 days and spores were harvested by adding water containing 0.01% tween80 (Merck). Mold spores were stored in 20 % glycerol + water and tween80 at -80 °C until use.

2.3 Culture conditions

CDIM was used for growth of Lb. paracasei DGCC 2132 and antifungal activity tests. The medium was prepared as described by Aunsbjerg et al. (unpublished results). CDIM (200 mL) was inoculated with Lb. paracasei DGCC 2132 (10⁷ CFU/mL) in 250 mL blue cap flasks and fermented at 37 °C for 22 hours to obtain a Cell containing ferment (C-ferment). pH was measured continuously in batches every 15 minutes during fermentation (Cinac, Alliance Instruments, Frepillon, France). All batches were made in triplicates. Cell Free ferments (CF-ferments) were prepared by centrifugation of C-ferment (5,000 x g, 15 min at 5 °C) followed by filtration of supernatant through a 0.45 µm filter (Frisenette, Aps). Un-inoculated CDIM kept at 37 °C for 22 hours and acidified with lactic acid to pH 4.5 was used as reference (REF). Plates with REF, C-ferment and CF-ferment used for antifungal activity tests were made by mixing with agar (1%).

2.4 Antifungal activity test

Antifungal activity was tested by spotting 20 µL of spore dilution (10⁵ spores/mL) of each mold in triplicates on plates of REF, C-ferment and CF-ferment and incubating at 25 °C for 9 days. Mold growth was documented by recording and analyzing multispectral images with the objective of quantifying area of the mold colonies.
Contribution of volatiles to antifungal activity was assessed in a "plate-on-plate" test system without direct contact between molds and C-ferment. A REF plate was spotted with 20 µL of a mold spore dilution (10^5 spores/mL). On top of the REF plate a C-ferment plate or a REF plate (control) was placed upside down and sealed with Parafilm® M. The inhibitory activity was assessed by growth on REF plates after 4 days of incubation at 25 °C.

2.5 Acquisition and Analysis of Multispectral Images
A VideometerLab 2 spectral imaging instrument (Videometer A/S, Hørsholm, Denmark) was used to record objective and reproducible images of the petri dishes with spotted mold. Images were recorded after 2, 3, 4, 5, 6 and 9 days of incubation. To record the multispectral images, the sample was placed inside the sphere of the instrument (Ulbricht sphere) where diffused light from light emitting diodes (LEDs) was provided at 18 different wavelengths, ranging from 375 to 970 nm. A single-channel image was recorded for each wavelength. The size of all the acquired images was 2056x2056x18. The lid of the petri dishes was removed prior to image acquisition to avoid reflection.

The images were subsequently analyzed using PCluster, an in-house MATLAB Graphical User Interface (GUI) developed by Ebrahimi et al. (unpublished results). PCluster is specifically designed for Penicillium molds, for which the colonies are composed of white and green segments; however, its main idea and the concept can be used for quantifying all types of molds. PCluster, clusters the pixels in the multispectral images with the objective of quantifying mold growth. The outputs of PCluster are the size (in pixels unit) of the green and white segments of the mold colonies and their average spectra. The quantification of mold growth was based on the size of the colonies by summing up the total number of pixels in each colony.

2.6 Analysis of Volatiles by Gas Chromatography Mass Spectrometry
Volatiles were measured by headspace Gas Chromatography Mass Spectrometry (GC-MS) configured for two types of headspace sampling. For qualitative screening and semi-quantitative work, headspace solid-phase micro extraction (HS-SPME) was employed. For quantitative purposes, static headspace sampling (SHS) was used.

Using the two methods, three types of samples were analyzed, liquid fermentations in CDIM (SPME+SHS), liquid fermentations in milk (SPME) and solid agar plugs (SPME+SHS). Real-time development of volatiles during fermentation in liquid samples (CDIM/milk) was monitored by
having a set of subsamples of the inoculated medium placed in the GC auto sampler, and the
headspace was sampled alternately from the vials. The development of volatiles in C-ferment or
volatiles present in CF-ferment was monitored by daily sampling of agar plugs from plates placed
in an incubator. The samples were stored in the freezer until the end of the study.

**Liquid calibration** solutions were prepared by acidifying CDI with DL-lactic acid to pH 4.5 and
adding diacetyl in the concentrations 0, 10, 25, 50, 100 and 200 µg/mL. Calibration solutions were
stored for a maximum of 24 hours at 5 °C. Aliquots of 2 ml_ of both calibration solutions and CDI
inoculated with *Lb. paracasei* DGCC 2132 were transferred to 20 ml_ headspace vials and sealed.

Samples were maintained at 37°C during headspace sampling and in between analyses by SHS
GC/MS (Table A.1).

Table A.1 Instrumental conditions for headspace sampling and analysis by solid phase
microextraction (SPME) and static headspace (SHS) GC/MS methods.

<table>
<thead>
<tr>
<th>Method</th>
<th>SPME GC/MS</th>
<th>SHS GC/MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature in auto sampler</td>
<td>Fermentations: 37°C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Agar plug samples: 5°C</td>
<td></td>
</tr>
<tr>
<td>Extraction temperature and time</td>
<td>37°C for 900 seconds with agitation</td>
<td></td>
</tr>
<tr>
<td>Incubation temperature and time</td>
<td></td>
<td>Fermentations: 37°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Agar plug samples: 50°C for 1200 seconds with agitation</td>
</tr>
<tr>
<td>Injection mode and</td>
<td>Splitless at 280°C</td>
<td>Split, split ratio 5:1 at 260°C</td>
</tr>
<tr>
<td>temperature</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Injection device and conditions</td>
<td>SPME Fiber: 85 µm Carboxen/polydimethyl/siloxane</td>
<td>2.5 mL syringe kopt at 110°C, 500 µl injection volume</td>
</tr>
<tr>
<td></td>
<td>(Supelco, Bellafonte, PA), Desorption time 30 seconds</td>
<td></td>
</tr>
<tr>
<td>Column</td>
<td>Agilent J&amp;W (Santa Clara, CA)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GC column DB-1701 (cross-linked and surface bonded</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14% cyanopropyl-phenyl/86% dimethyl/polysiloxane 60 m,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.25 mm ID, with a film thickness of 1 µm</td>
<td></td>
</tr>
<tr>
<td>Carrier gas and initial flow</td>
<td>He, 2 mL/min</td>
<td></td>
</tr>
<tr>
<td>Temperature programming</td>
<td>Hold for 1 min at 35°C, then 10°C/min to 45°C and</td>
<td>Hold for 2 min at 60°C, then 10°C/min to 110 and</td>
</tr>
<tr>
<td></td>
<td>ending with 25°C/min to 240°C.</td>
<td>ending with 20°C/min to 220 which is held for 2 min.</td>
</tr>
<tr>
<td>MSD transfer line temperature</td>
<td>260°C</td>
<td></td>
</tr>
<tr>
<td>MSD source temperature and</td>
<td>230°C and 70 eV</td>
<td></td>
</tr>
<tr>
<td>ionization voltage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solvent delay</td>
<td>0.7 min</td>
<td>2.0 min</td>
</tr>
<tr>
<td>Scan range, m/z</td>
<td>40-210</td>
<td>29-300</td>
</tr>
</tbody>
</table>
**Solid calibration** plates with diacetyl were prepared by acidifying CDIM with DL-lactate to pH 4.5 and adding diacetyl in the concentrations 0, 10, 30 and 90 µg/g. Plates were made by mixing with agar (1%). Plugs were taken from a sample plate (C- or CF-ferment) or a calibration plate by using an inverted sterile 1000 µl pipette tip (BioHit, Sartorius, Helsinki Finland) with vacuum suction. The plug was transferred to a 20 ml headspace vial, sealed and frozen at -18 °C. Collected agar plugs were stored at -18 °C for a maximum 14 days prior to analysis by SHS GC/MS.

Headspace analyses were performed using a CTC PAL autosampler (CTC Analytics, Zwingen, Switzerland) mounted on an Agilent 6870 GC coupled to Agilent 5973N single quadrupole MSD (Agilent Technologies, Waldbronn, Germany). The CTC PAL could be configured for both solid phase microextraction headspace analysis and static headspace analysis. Both the CTC pal and Agilent GC/MS were controlled by Agilent MSD Chemstation E. 02.00.49. Details on SPME and SHS instrumental conditions are listed in table A.1.

2.7 **Antifungal activity of diacetyl and acetoin**

 Antifungal activity of different concentrations of diacetyl and acetoin in CDIM (0, 10, 45, 60, 75 and 200 µg/mL) was tested at high (6.5) and low (4.5) pH, respectively. DL-lactic acid or HCl were used to acidify CDIM to pH 4.5. Plates were prepared by mixing with agar (1%) after addition of diacetyl and acids. Antifungal activity of different concentrations of diacetyl in milk and yogurt (0, 45, 75 and 200 µg/mL) was tested at pH 4.5 (acidified with DL-lactic acid). Plates of CDIM, milk and yogurt were spotted with 20 µL of spore dilution (10^5 spores/mL) of each mold in triplicates and incubated at 25 °C for up to 20 days.

2.8 **Reduction of inhibition by addition of alpha-acetolactate decarboxylase**

Diacetyl is formed from a spontaneous chemical oxidation and decarboxylation of the metabolic intermediate alpha-acetolactate (AL) formed by pyruvate (Fig. 1G.1). Alternatively, AL can be converted to acetoin by alpha-acetolactate decarboxylase (ALDC) (Kleerebezemab, Hols, & Hugenholtz, 2000; von Wright & Axelsson, 2011). To promote formation of acetoin from AL rather than diacetyl to reduce inhibitory effect, 400 µg/mL ALDC was added to CDIM prior to inoculation of *Lb. paracasei* DGCC 2132 (10^7 CFU/mL). Plates of C-ferment with and without added ALDC was made by mixing with agar (1%). Antifungal activity was tested against *P. solitum* DCS 302 as described in section 2.4.

2.9 **Production of volatiles in yoghurt with and without Lb. paracasei DGCC 2132**
UHT-milk (MILSANI®) at 5 °C was inoculated with 10 DCU/100 L YO-MIX™ 410 starter culture (DuPont Nutrition Biosciences Aps, Denmark) and with 10 DCU of both starter culture and $5 \times 10^6$ CFU/mL Lb. paracasei DGCC 2132. After mixing, $6 \times 5$ mL aliquots were pipetted to 20 mL headspace vials and capped. The vials were placed in an autosampler thermostated at 37°C. The headspace of the vials was sampled alternately with a cycle time of 20 min and analyzed by HS-SPME-GC/MS as described in section 2.6.

3. Results

3.1 Antifungal activity of bacterial ferment with and without cells

The antifungal activity of C-ferment and CF-ferment of Lb. paracasei DGCC 2132 was tested in CDIM against two indicator molds, P. solitum DCS 302 and Penicillium sp. DCS 1541. The C-ferment showed antifungal activity towards both molds, with Penicillium sp. DCS 1541 being more sensitive while the CF-ferment had little effect on the indicator mold growth.

In the plate-on-plate test system, with no direct contact between C-ferment and indicator molds, both molds were inhibited for 4 days (data not shown), strongly indicating the inhibitory role of some volatiles.

3.2 Diacetyl production and inhibition by C-/CF-ferment

Chemical analysis of the headspace of CDIM during fermentation with Lb. paracasei DGCC 2132 identified diacetyl as the primary volatile compound measured. Production of diacetyl was detected within the first few hours of fermentation with an increased production rate after 20 hours of fermentation (Fig. 1G.2). Prolongation of fermentation time increased diacetyl concentration further (data not shown).

To test if decreased antifungal activity of CF-ferment compared to C-ferment was due to difference in the volatile content, the diacetyl was measured in plugs from C-ferment as well as CF-ferment plates after 0, 1, 2, 4, 6, and 9 days of incubation at 25 °C. Diacetyl concentration increased in C-ferment from 58 to 74 µg/g after 1 day of incubation and then decreased upon further incubation. Diacetyl concentration in the CF-ferment was 9 µg/g at all days (Fig. 1G.3a).

Growth of the two molds was quantified based on the size of the colonies calculated from the multispectral images of REF, CF-ferment and C-ferment during incubation (Fig. 1G.3b and 1G.3b). For both molds, growth on CF-ferment were similar to the un-inoculated and acidified REF, and plates with CF-ferment were overgrown after 5 and 6 days for Penicillium sp. DCS 1541 and P.
solitum DCS 302, respectively. In contrast, the presence of LAB cells in C-ferment caused a delay in the onset of growth until day 3 for \textit{P. solitum} DCS 302 and until day 4 for \textit{Penicillium} sp. DCS 1541, as well as a markedly reduced growth rate of \textit{Penicillium} sp. DCS 1541.

3.3 Antifungal activity of diacetyl and acetoin
Diacetyl was added to CDIM in concentrations corresponding to those produced by \textit{Lb. paracasei} DGCC 2132 as well as in higher concentrations as used in previous mold inhibition studies (Jay, 1982b). Increasing diacetyl concentration correlated with increased inhibition of indicator molds, with \textit{Penicillium} sp. DCS 1541 being the most sensitive target organism. At diacetyl concentrations corresponding to the amount present in C-ferment plates at day 0 (-60 µg/mL) a marked inhibition of mold growth was observed up to 3 days of plate incubation whereafter the mold started growing (Fig. 1G.4). At 75 µg/mL diacetyl mold growth was suppressed for up to 5 days (data not shown).

\textit{Penicillium} sp. DCS 1541 was more sensitive to diacetyl at low pH, whereas \textit{P. solitum} DCS 302 was more sensitive at the high pH. The use of HCl to acidify CDIM showed same results as lactic acid (data not shown). If high amounts (200 µg/mL) of diacetyl were added to either CDIM, milk or yogurt, \textit{P. solitum} DCS 302 did not grow until day 14, and no growth of \textit{Penicillium} sp. DCS 1541 was observed after 20 days (data not shown).

Acetoin did not show antifungal activity towards the two molds at any of the concentrations tested (data not shown).

3.4 Reduction of inhibition by addition of alpha-acetolactate decarboxylase
ALDC has been used in the brewing industry to remove diacetyl which is considered an off flavor in beer (Yamano et al., 1995). ALDC was added to CDIM prior to inoculation with \textit{Lb. paracasei} DGCC 2132 in order to promote formation of acetoin from AL and thereby decreasing the formation of diacetyl during fermentation. Addition of ALDC did not influence growth of molds on uninoculated plates of CDIM (data not shown). With increasing concentrations of ALDC added to the C-ferment, the inhibitory effect of the ferment decreased accordingly (Fig. 1G.5).

3.5 Production of volatiles in yogurt with and without \textit{Lb. paracasei} DGCC 2132
The volatiles in the headspace of yogurt with and without added \textit{Lb. paracasei} DGCC 2132 were measured by SPME-GC/MS in order to follow their formation when the antifungal \textit{Lb. paracasei} DGCC 2132 was added to yogurt. A number of volatiles were detected in both types of yogurt (Fig. 1G.6). Addition of \textit{Lb. paracasei} DGCC 2132 significantly increased the formation of diacetyl and
acetoin. Besides diacetyl and acetoin, acetic acid increased slightly in the yoghurt co-fermented with *Lb. paracasei* DGCC 2132 and other differences were observed for the remaining volatiles such as 2,3 pentadione and butanoic acid reflecting the higher complexity in yogurt.

3.6 Antifungal activity of diacetyl in yoghurt and acidified milk

The antifungal activity of yogurt and milk with added diacetyl against *P. solitum* DCS 302 and *Penicillium* subsp. DCS 1541 supported the results obtained in CDIM (Fig. 1G.7). Similar results were observed for milk acidified with lactic acid and added diacetyl (results not shown).

4. Discussion

Many earlier studies of bacterial cultures with antifungal effect have focused on identification of potent antifungal compounds in cell free ferments (Sarah Crowley, Mahony, & van Sinderen, 2013; Magnusson & SchnGrer, 2001; Rouse, Harnett, Vaughan, & van Sinderen, 2008; Schwenninger et al., 2008). However, loss of antifungal activity of culture ferments was observed after cell removal, an observation also made by (Schwenninger & Meile, 2004). Since antifungal activity could be observed in a plate-on-plate test with no direct contact between C-ferment and mold, it seemed likely that volatiles were involved in the antifungal activity. Moreover it was found that most of the volatile diacetyl produced during fermentation disappeared when preparing cell free ferments. Volatiles are easily lost in the steps involved in cell removal e.g. centrifugation, filtration and heating of ferment so, if this is not taken into account, the contribution of volatiles to antifungal activity could be overlooked or underestimated.

In CDIM, the main volatile compound measured in the headspace of C-ferment during fermentation was diacetyl. Diacetyl production increased rapidly at pH below 5 and increasing fermentation time was found to further increase diacetyl (data not shown). This is in agreement with a study on *Lb. casei* by Branen and Keenan (1971) who observed a rapid increase in diacetyl content at pH below 5.5 with the highest production measured between pH 4.5 and 5.5. In CDIM, the concentration of diacetyl was 22 µg/g after the initial 22 hours of fermentation and it rose to 58 µg/g during the preparation of C-ferment plates indicating continuous production of diacetyl by *Lb. paracasei* DGCC 2132. Besides diacetyl, traces of acetoin were observed for the CDIM fermentation.

When levels of diacetyl were added to CDIM corresponding to those found in the plates of C-ferment (-60 µg/g) and CF-ferment (-10 µg/g), inhibition for up to 3 days and no antifungal activity, respectively, was observed indicating that the high diacetyl concentration in C-ferment was the main cause of the inhibitory activity (figure 1G.4).
Diacetyl is a common volatile metabolite in fermented milk products (Ott, Germond, & Chaintreau, 2000) with reported concentrations between 4.5-27 µg/g depending on the product (Rincon-Delgadillo, Lopez-Hernandez, Wijaya, & Rankin, 2012). Yogurt is a more complex medium which, in itself, contains numerous volatile compounds apart from diacetyl after fermentation. When Lb. paracasei DGCC 2132 was added together with the starter culture, some of the easily identifiable changes were increased amounts of diacetyl and acetoin but also some relative increases in potential antifungal compounds like 2,3 pentadione, acetic acid, and butanoic acid were observed. Co-fermentation of the Lb. paracasei DGCC 2132 strain with a yogurt starter culture demonstrated a marked increase in both diacetyl and acetoin as compared to yogurt without Lb. paracasei DGCC 2132. Furthermore, addition of diacetyl as a single compound to yogurt and acidified milk showed inhibitory activity against the two molds with results similar to CDIM.

The antifungal properties of diacetyl as an isolated compound were assessed as early as 1941 towards several molds including Penicillium sp. and Fusarium sp. with inhibition observed at concentrations above 86 µg/mL (Lagoni, 1941). Other studies showed sensitivity of molds and yeast to diacetyl concentrations of 100 and 200 µg/mL diacetyl, respectively (Jay, 1982a, 1982b), with increased activity at low pH for some molds (Jay, 1982b). The mechanism behind the inhibition of mold growth has not been investigated and little is known of interactions with other compounds. Here, it has been found that the Penicillium strains were totally inhibited for up to 5 days in CDIM with added diacetyl concentrations above 75 µg/mL and, furthermore, that the interaction with pH was strain dependent.

Suomalainen and Mayra-makinen (1999) found that levels of diacetyl and acetic acid increased in a bacterial culture mix with activity against yeast and Bacillus spp. While the amount of acetic acid doubled, the diacetyl levels increased in yogurt and in quark from <0.5 to 24 and from 0.6 to 49 µg/mL, respectively. Although the inhibitory mechanism was not investigated, it was stated, based on the data from Jay (1982b), that the diacetyl levels found would be insufficient to explain all the antifungal activity. Similar statements are found in several reviews on antifungal LAB (Caplice & Fitzgerald, 1999; Schnurer & Magnusson, 2005) and a recent review on antifungal compounds produced by LAB does not mention diacetyl at all (S. Crowley et al., 2013). This may reflect that it has been difficult to estimate the effect of LAB-produced diacetyl in microbial interactions.

Apart from the loss of volatiles in many sampling operations, some caution in the interpretation of volatile production from fermentations should be exerted since the equilibrium between the
headspace and liquid phase is influenced by temperature, pH and other factors (Pawliszyn, 1997). In this Example, two headspace sampling techniques were employed to follow the dynamics of volatiles independent of the matrix. For initial profiling of the headspace, SPME was used as an unbiased, sensitive screening technique and SHS was used for absolute quantification of e.g. diacetyl. There may, however, still be changes in compounds which are not readily detected. Differences in diacetyl production can be caused by absence or suppression of ALDC, resulting in formation of diacetyl instead of acetoin from accumulated AL (Kleerebezem et al., 2000). Another factor could be absence or decreased activity of the acetoin reductase (AR), which reduces diacetyl to acetoin. ALDC was added to CDIM prior to inoculation with Lb. paracasei DGCC 2132 in order to test if the low antifungal activity observed in CF-ferment was caused by a decrease in diacetyl content after cell removal. Addition of ALDC markedly decreased antifungal activity of the C-ferment, although the activity was not completely abolished. The expected higher amounts of acetoin caused by the ALDC addition could not explain the remaining antifungal activity of C-ferment when ALDC was added, since acetoin showed no antifungal activity at the relevant concentrations. Jay et al., (1983) also found low antifungal activity of acetoin. The observed antifungal activity in C-ferment with added ALDC might be a result of antifungal metabolites other than diacetyl causing the remaining effect. Another reason could be that ALDC did not efficiently remove all diacetyl.

Lactic acid bacteria known as diacetyl producers have been associated with antifungal properties, but no causal link between antifungal effect of these cultures and their diacetyl production has previously been documented. Here, the production of volatile diacetyl by Lb. paracasei DGCC 2132 is linked with inhibition of Penicillium strains thereby pointing at a previously overlooked contribution to the anti-mold effect of LAB. A multitude of bacterial metabolites, both volatile and non-volatile, may influence potential mold growth and the interactions can be complex and depending on many factors. The use of cultures with continuous production of diacetyl and other synergistically active compounds may have a strong potential as clean label ingredients in products prone to fungal spoilage but further knowledge is needed to optimize cultures for specific products and spoilage organisms.

References for Example 1G


Strom, K., Sjogren, J., Broberg, A., & Schnurer, J. (2002). Lactobacillus plantarum MiLAB 393 produces the antifungal cyclic dipeptides cyclo(L-Phe-L-Pro) and cyclo(L-Phe-trans-4-OH-L-Pro) and 3-phenyllactic acid. Applied and Environmental Microbiology, 68, 4322-4327. doi:10.1128/AEM.68.9.4322


Example 1H - Metabolic Footprinting for Investigation of antifungal properties of Lactobacillus paracasei

Lactic acid bacteria with antifungal properties are applied for bio-preservation of food.

For understanding the antifungal mechanism, there is an ongoing search for the bioactive molecules. With focus on formed metabolites, bioassay guided fractionation and comprehensive screening have identified compounds as antifungal. Although active, the compounds have been found in concentrations too low to account for the antifungal effect.
The antifungal effect is hypothesized as being the result of bacterial fermentation with formation of metabolites and consumption of nutrients, *i.e.* the composition of the exometabolome. To build a more comprehensive view of the chemical changes induced by bacterial fermentation and the effects on mold growth, a strategy for correlating the exometabolomic profiles to mold growth was applied.

The antifungal properties were measured as mold growth of two *Penicillium* strains on cell free ferments of three strains of *Lactobacillus paracasei* pre-fermented in a chemically defined medium.

Exometabolomic profiling was performed by reversed phase liquid chromatography in combination with mass spectrometry in electrospray positive and negative modes.

By multivariate data analysis, the three strains of *Lb. paracasei* were readily distinguished by the relative difference of their exometabolomes, both in terms of formation of metabolites and consumption of nutrients. The relative differences correlated to the relative growth of the two *Penicillium* strains.

Metabolic footprinting proved as a supplement to bioassay guided fractionation for investigation of antifungal properties of bacterial ferments. Additionally, three previously identified and three novel antifungal metabolites from *Lb. paracasei* and their potential precursors were detected and assigned using the strategy.

**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation (ArAA)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ArAA</td>
<td>Aromatic amino acid</td>
</tr>
<tr>
<td>BCAA</td>
<td>Branched chain amino acid</td>
</tr>
<tr>
<td>BPC</td>
<td>Base peak chromatogram</td>
</tr>
<tr>
<td>CDIM</td>
<td>Chemically defined interaction medium</td>
</tr>
<tr>
<td>CF</td>
<td>Cell free ferment</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>FID</td>
<td>Flame ionization detector</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>id</td>
<td>Internal diameter</td>
</tr>
<tr>
<td>ID</td>
<td>Inhibition degree</td>
</tr>
</tbody>
</table>
**INTRODUCTION**

The use of lactic acid bacteria as an additional hurdle to help controlling fungal spoilage is widely applied in food products. Much work has been invested in identifying the antifungal metabolites produced by lactic acid bacteria. Focus has been on bioassay guided fractionation [Strom 2002], i.e. with a hypothesis of finding one or several compounds responsible for the antagonistic effect e.g. [1-4]. Over time, numerous compounds have been assigned as antifungal as compiled in recent comprehensive reviews [5, 6]. Although the compounds have been shown to be active, the level produced in biological system has been below minimal inhibitory concentration (MIC) [7-9]. The use of combinations of metabolites in some studies have indicated that the antifungal effect of ferment could be due to synergistic effects between metabolites [9-11]. The strategy of bioassay guided fractionation is especially suited for detecting one or few potent antagonistic compounds, but has shortcomings when the effect is composed.

Recent studies have employed more comprehensive analytical screening strategies with a minimum of sample preparation or fractionation [12, 13]. The workflow excludes sample preparation steps like solid phase extraction (SPE), where loss of bioactive compounds have been observed [12, 14]. Instead, untreated or pre-concentrated cell free ferments (CFs) are studied on
hyphenated high performance chromatography - high-resolution mass spectrometry analytical platforms. This enables recognition and quantification of multiple known compounds [12].

Within the potential of state-of-the-art analytical platforms, the analytical coverage can be expanded to go beyond the recognized compounds to include all available signals. Then, the study becomes an untargeted or data-driven study of the CF or exometabolome of lactic acid bacteria. Studies of an extensive range of extracellular metabolites including residual nutrients have been defined as exometabolomic footprinting [15]. Exometabolomic studies hold potential for new learning related to the antifungal properties of lactic acid bacteria. The mode of action of a new antibiotic compound was elucidated using this strategy [16]. Paczia et al. (2012) demonstrated the dynamics of extended overflow metabolism showing passive and active transportation of central metabolic intermediates to the extracellular environment during batch fermentations [17]. The change in metabolism of branched chain amino acids (BCAA) as stress induced by acidic conditions was examined for Lactobacillus sanfranciscensis [18].

By adopting the workflow of untargeted metabolomics, the present Example aimed at providing a supplementary tool for investigating the antifungal properties of three strains of Lactobacillus paracasei. The three Lb. paracasei strains were fermented in a chemically defined interaction medium (CDIM). The CFs were subjected to metabolic footprinting and tested for effect on growth of two Penicillium indicator strains. With this approach, the dynamics for both nutrient availability and formation of metabolites was taken into account. The data driven approach applied multivariate methods for identifying biomarkers from correlations between compounds and biological effect. The correlations were converted into hypotheses for targeted studies and tested in model systems to test for causal links between compound concentrations and biological activity.

MATERIALS AND METHODS

Chemicals

Solvents and chemicals, including formic acid, acetic acid, propionic acid, butanoic acid, 2-ethylbutanoic acid, lactic acid (80%), 2-hydroxy-3-methylbutanoic acid (= '2-hydroxy-isovaleric acid'), 2-hydroxy-3-phenylpropanoic acid (= 'phenyllactic acid'), 2-hydroxy-3-(4-hydroxyphenyl)propanoic acid (= '4-hydroxyphenyl)lactic acid'), 2-hydroxy-3-(1 H-indol-3-y1)propanoic acid (= 'indolelactic acid'), 2-hydroxy-4-methylpentanoic acid (= '2-hydroxyisocaproic acid'), 2-hydroxy-(4-methylthio)butanoate Calcium salt, inosine, hypoxanthine, L-phenyl-D-\(\varepsilon\)-alanine, sodium chloride, sulfuric acid, diethyl ether, acetonitrile and ethanol were purchased from Sigma-Aldrich (Schnelldorf, Germany) with purity in excess of 95% or as pro analysis quality unless
otherwise specified. All water employed was of freshly prepared Milli-Q quality (Merck Millipore, Billerica, MA, USA).

**Microbial strains**

All strains used in these studies were supplied by DuPont Nutrition Biosciences ApS. The strains and their growth conditions are listed in Table 4H.1.

<table>
<thead>
<tr>
<th>Microbial strain</th>
<th>Short name</th>
<th>Growth medium</th>
<th>Incubation temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lactobacillus paracasei</em> DGCC 2132</td>
<td>LAB A</td>
<td>MRS</td>
<td>37°C</td>
</tr>
<tr>
<td><em>Lactobacillus paracasei</em> DGCC 11287</td>
<td>LAB B</td>
<td>MRS</td>
<td>37°C</td>
</tr>
<tr>
<td><em>Lactobacillus paracasei</em> DGCC 695</td>
<td>LAB C</td>
<td>MRS</td>
<td>37°C</td>
</tr>
<tr>
<td><em>Penicillium solitum</em> DCS 302</td>
<td>DCS 302</td>
<td>MEA</td>
<td>25°C</td>
</tr>
<tr>
<td><em>Penicillium</em> spp. DCS 1541</td>
<td>DCS 1541</td>
<td>MEA</td>
<td>25°C</td>
</tr>
</tbody>
</table>

**Preparation of *Lactobacillus paracasei* cell free ferments (CFs)**

The three strains of *Lb. paracasei* were pre-inoculated in MRS and incubated at 37°C overnight. The cells were harvested by centrifugation followed by washing of cells twice in 0.9% NaCl. Blue cap bottles (250 ml) with CDIM were inoculated with washed cells of the three strains to an OD₅₅₀nm of 0.05. Five biological replicates were made of each batch. Batches were placed in a 37°C water bath with CinAC pH electrodes monitoring pH every 15 minutes throughout fermentation. Aliquots were taken from each batch after 65 hours of fermentation and placed on ice. The biomass of a 45 ml sample aliquot was determined as the dry mass after filtration through Advantec GB140 (Toyo Roshi Kaisha, Ltd, Japan). CFU/mL and OD₅₀₀ nm was in addition measured after 65 hours of fermentation.

Cell free ferments (CFs) were prepared by centrifuging at 4500 g for 10 min at 0°C (Heraeus XR3 Multifuge, Thermo Fisher Scientific, Waltham, MA, USA) and the supernatant was filtered through a sterile 0.45 μm pore size filter (cellulose acetate membrane, Q-Max, Frisenette Aps, Knebel, Denmark). Aliquots (1000 μl) of the filtrate were distributed into eppendorf tubes for liquid chromatography/mass spectrometry (LC/MS) analysis, frozen on dry ice and stored at -80°C.
Headspace Solid-Phase Microextraction Gas chromatography Mass Spectrometry for Monitoring Fermentation Volatiles

The ferments were analyzed for volatile profile by headspace solid phase microextraction gas chromatography with mass spectrometric detection (HS SPME-GC/MS). The method was as described in Aunsbjerg et al. (Aunsbjerg, Honore, Vogensen, Knöchel, Development of a chemically defined medium for studying foodborne bacterial-fungal interactions, submitted for publication).

In brief, the analysis was performed using a CTC PAL autosampler (CTC Analytik, Zwingen, Switzerland) mounted on an Agilent 6870 GC coupled to Agilent 5973N single quadrupole MSD (Agilent Technologies, Waldbronn, Germany). Both the CTC pal and Agilent GC/MS were controlled by Agilent MSD Chemstation E. 02.00.49.

Immediately after inoculation, an aliquot of 2 ml were transferred to 20 ml headspace vials. The samples were placed on the autosampler thermostated to 37°C and the headspaces were alternately sampled by SPME with an extraction time of 900 seconds.

The GC was mounted with a 60m x 0.25mm internal diameter (id) Agilent J&W (Santa Clara, CA) GC column DB-1701 (cross-linked and surface bonded 14% cyanopropyl-phenyl/86% dimethylpolysiloxane) 60 m, 0.25 mm id, with a film thickness of 1 µm. The carrier gas was helium flowing at 2.0 ml/minutes. The SPME fiber (85 µm Carboxen/polydimethylsiloxane (Supelco, Bellafonte, PA) was desorbed for 30 seconds with a split ratio of 1:5 split/split less injector kept at 260°C. The oven initially was held at 60°C for 2 min then temperature was increased to 110°C with a rate of 10°C per minute and then to 240°C with 20°C per minute. The transfer line and MS was held at 260°C and 230°C, respectively. The ionization voltage was 70 eV, and MS scan range was m/z 29-300.

Mold growth assay
Mold growth on Lb. paracasei CF: CFs and un-inoculated CDIM acidified to pH 4.5 with DL-lactic acid (REF) were mixed with agar (1%) and poured into petri dishes. Antifungal activity of 3 of 5 biological replicates of the three Lb. paracasei CF and REF was tested against P. solitum DCS 302 and Penicillium sp. DCS 1541. The molds were spotted (20 µl of 10^5 spores/ml) on plates in triplicates and incubated at 25°C for 2-5 days.
Mold growth on simulated ferments: The effect of pH, identified 2-hydroxy acids (Table 1H.8) and absence of glucose was tested against the two indicator molds on plates of simulated CDIM ferments (SIMLAB). CDIM was acidified with DL-lactic acid to pH corresponding to average pH of LAB A, LAB B and LAB C, respectively after 3 days of fermentation (Table 1H.3). Identified and quantified compounds were added in the concentrations produced by the three strains (Table 1H.8). Batches corresponding to ferment of LAB C were made with and without glucose (Glc) as in CDIM. CDIM pH adjusted with DL-lactic acid without added compounds were used as references. Triplicate determinations were made. An overview of batches of simulated ferments is listed in Table 1H.5. Antifungal activity of simulated ferments was tested as described for *Lb. paracasei* ferments.

### Table 1H.5 Overview of batches of simulated ferments

<table>
<thead>
<tr>
<th>Short name</th>
<th>pH of LAB A</th>
<th>2-hydroxy acids</th>
<th>Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIMLAB A with acids</td>
<td>pH of LAB A</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>SIMLAB B with acids</td>
<td>pH of LAB B</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>SIMLAB C with acids</td>
<td>pH of LAB C</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>SIMLAB C with acids excl Glc</td>
<td>pH of LAB C</td>
<td>x</td>
<td>-</td>
</tr>
<tr>
<td>SIMLAB A without acids</td>
<td>pH of LAB A</td>
<td>-</td>
<td>x</td>
</tr>
<tr>
<td>SIMLAB B without acids</td>
<td>pH of LAB B</td>
<td>-</td>
<td>x</td>
</tr>
<tr>
<td>SIMLAB C without acids</td>
<td>pH of LAB C</td>
<td>-</td>
<td>x</td>
</tr>
<tr>
<td>SIMLAB C without acids excl Glc</td>
<td>pH of LAB C</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Multispectral images of *Lb. paracasei* ferments and simulated ferments were recorded on day 2-5 on a daily basis using a VideometerLab 2 spectral imaging instrument (Videometer A/S, Hørsholm, Denmark). Diffused light from light emitting diodes (LEDs) were provided at 18 different wavelengths, ranging from 375 to 970 nm. Single-channel images were recorded for each of the 18 wavelengths (from 375 to 970 nm). The instrument was mounted with a JAI (JAI A/S, Valby Denmark) model BM-500GE 32 bit camera with a resolution of 2056 x 2056 resulting in a pixel size of 45.8 μm. The lid of the petri dishes was removed prior to image acquisition to avoid reflection. The multispectral images were subsequently analyzed using a modified version of PCluster, a GUI developed under MATLAB environment (Version 2012b, MathWorks, Inc., Natick, MA, USA) by Ebrahimi et al. (Ebrahimi, van den Berg, Aunsbjerg, Honore, Benfeldt, Jensen and Engelsen: Quantitative determination of mold growth and inhibition by multispectral imaging, submitted for publication). In the modified version of PCluster, the pixels in the multispectral images are clustered with the objective of quantifying different segments of the mold colonies, and as a result, quantifying mold growth and mold color of colonies. The outputs of the analysis are number of the white and green segments in the colonies and their average spectra.

**Minimal inhibitory concentrations of identified compounds**
The six identified 2-hydroxy acids (Table 1H.8) were tested for their minimal inhibitory concentration (MIC) against the two strains of Penicillium. Solutions of CDIM acidified with DL-lactic acid (pH 4.5) were prepared with 0 (REF), 0.1, 1, 5 and 10 mg/ml of all six compounds, respectively. The test solutions were distributed into a sterile flat-bottomed 96 well microplate (Fisher Scientific) and $10^4$ spores/ml of each Penicillium strain were added to the wells. Triplicate determinations were made. Microplates were incubated at 25°C for up to 48 hours. OD at start ($t_0$) and after 48 hours ($t_4$) was recorded at 600 nm using a Varioskan Flash (Thermo Fisher Scientific Oy, Finland).

The inhibition degree (ID) was calculated and evaluated as [9]:

$$ID = \frac{OD_{600}(test \ solution, \ t_4) - OD_{600}(test \ solution, \ t_0)}{OD_{600}(Reference, \ t_4) - OD_{600}(Reference, \ t_0)}$$

The minimal inhibition concentration (MIC 50) was defined as the concentration providing ID < 0.5 [9].

**Metabolic footprinting by Liquid Chromatography Mass Spectrometry (LC/MS)**

Frozen CFs were thawed in an Eppendorf Thermomixer Comfort (Eppendorf Nordic Aps, Horsholm, Denmark) for 10 minutes at 20°C with 750 rpm. A pooled sample (MIX) was prepared by sampling and mixing 200 μl of each CF included in the study.

Aliquots of 200 μl of the CF, the MIX sample or Milli-Q water (solvent blank) were diluted with 800 μl 0.1% v/v formic acid in water containing 0.06 mmol/l L-phenyl-D₅-alanine as internal standard (IS). The diluted solution was centrifuged at 13300 x g for 10 minutes prior to analysis (Spectrafuge Labnet International Inc. Edison, NJ, USA).

The LC/MS system was equilibrated with a minimum of ten replicate injections of the MIX sample prior to analyzing samples. Sample injections were performed in duplicate. Each set of replicates were placed in randomized brackets containing ten samples (representing two replicates of the five biological treatments) and one MIX sample. Each injection bracket was initiated by a solvent blank (Milli-Q water) treated as sample.

The LC/MS analysis was performed using an Agilent (Agilent Technologies, Waldbronn, Germany) modular 1290 ultra performance liquid chromatography (UPLC) instrument coupled to a Bruker (Bruker Daltonics, Billerica, MA) maXis 4G single quadrupole time-of-flight mass spectrometer (MS) via an electrospray interface. The UPLC was mounted with a Waters (Waters Corporation, Milford, Ma, USA) HSS T3, 2.1 x 150 mm id column packed with 1.8 μm particles. Mobile phases
were A) water/formic acid 1000/1 v/v and B) acetonitrile/formic acid 1000/1 v/v. Vials were kept at 5°C in the autosampler prior to injection of 10 µl. Elution was performed with a flow of 400 µl/min and a gradient starting at 0% B at t=0 and kept for 1.0 min, then to 100% B at 15 minutes and kept for 0.5 minutes. Then back to 0% B over 0.1 minutes and maintained for 4.4 minutes. The electrospray interface with nebulizer at 2.5 bar and dry gas at 9.0 L/min at 200°C was operated in both positive and negative mode (capillary voltage at 4000 V and 3200 V, respectively). Mass spectra in the range m/z 60 - 1250 were acquired with a frequency of 3 Hz. Spectra were saved as centroided. The mass to charge axis was calibrated with sodium formate clusters (solution of water/2-propanol/1 mol 1⁻¹ sodium hydroxide/formic acid 250/250/2.5/0.5 v/v/v/v) infused prior to each chromatographic run via a divert-valve-loop setup. The instrument was controlled using Bruker Daltonics micrOTOFcontrol version 3.1 and acquired data was handled with Data Analysis version 4.0 SP4.

Identification and quantification by LC/MS

Stock solutions of the individual standards (Table 1H.8) of 1 mg/ml were prepared. Dilution series of combinations of the stock solutions were prepared with concentrations of ca. 3, 10, 30, 100 µg/ml of each of the six 2-hydroxy acids. The dilution series were used for preparing calibration solutions and for spiking CFs of LAB C. Aliquots 400 µl of the dilution series were added to 200 µl of either 0.1% formic acid (calibration solutions) or the CF (spiked ferments) and additional 400 µl 0.1%v/v formic acid in water containing 0.12 mmol/l L-phenyl-D₅-alanine as IS. The samples were analyzed by the LC/MS conditions listed previously. Verification of identity by retention time assignment and mass spectrum of compounds was made by analyzing solutions of 0.1 mg/ml.

Quantification of organic acids by gas chromatography with flame ionization detection (GC/FID)

Aqueous stock solutions of standards were prepared containing the six hydroxy acids of interest (Table 1H.8) and the IS, 2-ethylbutanoic acid. Aliquots of standards (0.5-400 µl) or CF samples (100, 200, 300 µl) mixed with IS solution (50 µl) were derivatized with 2 ml ethanol in sulfuric acid (concentrated, p.a.) 150/50 v/v for 2 hours at 80°C. The solution was cooled to ambient temperature and 10 ml of a 10 w/w% sodium chloride in water was added. The combined solution was extracted with 2 ml diethyl ether for 30 minutes by vigorous shaking.

The diethyl ether extracts were analyzed on an Agilent (Agilent Technologies, Waldbronn, Germany) 6890N GC with flame ionization detector (FID). The Agilent GC was controlled by Agilent GC Chemstation rev. B.04.01 SP1. The GC was mounted with a 10m x 0.10mm id Quadrex
(Quadrex Corporation, Bethany, CT) 007-FFAP (nitroterephthalic acid modified polyethylene glycol polymer) column with a film thickness of 0.1 µm. The carrier gas was He flowing at 0.8 ml/min. The sample injection was 1 µl with split ratio of 1:100 in a split/split less injector kept at 250°C. The FID was held at 240°C. Two different temperature gradients were employed. For low boiling derivatives, the oven initially was held at 40°C for 2 minutes then temperature was increased to 240°C with a rate of 20°C per minute. For higher boiling derivatives, the oven start temperature held at 150°C for 2 minutes, and then was increased to 240° with a rate of 20°C/min to 240°C held for 10 minutes.

Data processing

Metadata like pH (from CINAC) and biomass as well as randomizing injection sequences was handled in Microsoft Excel 2007 (Microsoft).

Prior to feature extraction, chromatographic data of MIX samples (total ion chromatograms (TICs) and base peak chromatograms (BPCs)) were inspected visually for irregularities like drift in intensities and retention time.

Bruker raw data files were converted into mzXML files by Bruker CompassXport v.3.0.5 (Bruker Daltonics). Feature extraction made with MZmine2 [20]. Peak detection was based on an m/z tolerance of 0.001 Dalton (Da) or 5 ppm and a peak duration time range of 0.025 - 0.35 minutes. Chromatograms were deconvoluted using the 'local minimum search' algorithm, de-isotoped and peaks were aligned using the Join aligner algorithm. Peak lists were filtered using a criterion of minimum a feature being detected in five chromatograms (the data set held 5 biological replicates analyzed twice; technical duplicates). The peak list was gap filled and filtered for duplicate peaks with a retention time tolerance of 0.1 minutes and 0.001 Da or 5 ppm.

Multivariate data analysis was performed in MATLAB R2013a version 8.1.0.604, 64-bit (MathWorks, Natick, MA, USA) and PLS Toolbox version 7.3.1 (Eigenvector Research, Inc., Wenatchee, WA, USA).

Statistical analysis was performed in Statgraphics Centurion XVI, version 16.1.17, 64 bit (Statpoint Technologies Inc., Warrenton, VA, USA).

RESULTS

Characteristics of Lb. paracasei Fermentations and Cell Free Ferments
The three *Lb. paracasei* strains were fermented in parallel for 65 hours (end of fermentation). All three strains were diacetyl producing (data not shown). From an initial similar inoculation concentration (in terms of OD), two of the strains LAB B and LAB Cfermented to a lower pH than LAB A. Corresponding to the larger decrease in pH, the LAB B and LAB C strains generated significantly higher amount of biomass and higher cell counts than LAB A and LAB C (Table 1H.6).

**Table 1H.6 Characteristics of *Lb. paracasei* fermentations (average ± standard deviation)**

<table>
<thead>
<tr>
<th><em>Lb. paracasei</em> strain (Short name)</th>
<th>DGCC2132 (LAB A)</th>
<th>DGCC11287 (LAB B)</th>
<th>DGCC695 (LAB C)</th>
<th>Reference (REF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell count (log CFU/ml, n=3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Start of fermentation</td>
<td>6.8 ± 0.1</td>
<td>7.6 ± 0.1</td>
<td>6.9 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>End of fermentation</td>
<td>7.8 ± 0.1</td>
<td>9.4 ± 0.2</td>
<td>9.4 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>OD&lt;sub&gt;600 nm&lt;/sub&gt; (abs, n=5),</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Start of fermentation</td>
<td>0.052 ± 0.002</td>
<td>0.048 ± 0.002</td>
<td>0.050 ± 0.004</td>
<td></td>
</tr>
<tr>
<td>End of fermentation</td>
<td>0.50 ± 0.04</td>
<td>1.42 ± 0.02</td>
<td>1.50 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>Diacetyl production during fermentation</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>pH (n=5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Start of fermentation</td>
<td>6.55 ± 0.03</td>
<td>6.55 ± 0.03</td>
<td>6.56 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>End of fermentation</td>
<td>4.53 ± 0.08</td>
<td>3.84 ± 0.06</td>
<td>3.77 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>Biomass (g/l, n=5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>End of fermentation</td>
<td>0.15 ± 0.01</td>
<td>0.56 ± 0.02</td>
<td>0.65 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>Mold growth after three days at 25°C (10&lt;sup&gt;3&lt;/sup&gt; pixels, n=3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DCS 302</td>
<td>6.31 ± 0.25</td>
<td>5.19 ± 0.27</td>
<td>4.40 ± 0.18</td>
<td>7.38 ± 0.07</td>
</tr>
<tr>
<td>DCS 1541</td>
<td>6.70 ± 0.22</td>
<td>4.82 ± 0.01</td>
<td>4.51 ± 0.10</td>
<td>8.01 ± 0.25</td>
</tr>
</tbody>
</table>

The growth of the two indicator molds, *P. soillum* DCS 302 and *Penicillium* spp. DCS 1541 was tested on the agar plates of un-inoculated media at pH 4.5 and CFs of the three *Lb. paracasei* strains. On a daily basis, mold growth was monitored by multispectral imaging which was processed into a measure of the sum of white and green pixels. Mold growth was characterized by only white pixels on day two and from day three and onwards by both white and green pixels as outlined in supplementary material S1 (see Fig 1H.S1). From day three and onwards, the three *Lb. paracasei* CFs could be ranked according to the mold growth: REF > LAB A > LAB B > LAB C (Table 1H.3).
Metabolic footprinting by Liquid Chromatography Mass Spectrometry (LC/MS)

The chemical profiles of the Lb. paracasei exometabolomes (i.e. CFs) were generated by reversed phase-LC/MS via both electrospray ionization (ESI) positive (pos) and negative (neg) mode. The two ESI polarities were complementary in detecting both substrate components and metabolites as shown in Fig. 1H.1. Compounds with acidic protons, like carboxylic acids, were more prone to detection in ESI neg by formation of M-H ions. The most intense peaks of the ESI pos were amino acids, adenosine and adenine from the CDIM. The most polar amino acids (like arginine, asparagine, aspartic acid, glutamine, glutamic acid) were only slightly retained on the column and eluted within or just after the void volume.

Feature extraction using MZmine2 resulted in 977 features in ESI pos and 142 features in ESI neg mode. The metabolites for both polarities were normalized with the corresponding feature representing IS. Especially in ESI pos mode, compounds were represented with multiple features due to different adducts and in-source fragmentation. As an examples glucose was observed as [M+Na]⁺, [M+NH₄]⁺, [M+K]⁺, as [2M+Na]⁺ cluster and as corresponding in-source fragments after water loss. Adducts and clusters where designated "Glc" and in-source fragments "Glc"⁻. At least two sets of features represent the sum of positional isomers closely eluting, namely ‘Leu+Ile’ covering leucine and isoleucine and O H-Me-Pe’ covering 2-hydroxy-Y-methylpentanoic acid with Y being 3 or 4.

Principal component analysis (PCA) after mean centering and Pareto scaling grouped the samples according to their origin, i.e. un-inoculated CDIM (REF), the three types of ferments and a centered group of the pooled quality control sample (Supplementary material S2 - see Fig 1H.S2).

The technical replicates (n=2) were averaged for each biological replicate (n=5). For each of the two ESI modes, scores and loadings of PCAs of the averaged data are shown in fig. 1H.2.

The first principal component (PC) for both ESI pos and neg mode data corresponded to the classes observed given by both acidification and biomass production, i.e. grouping of LAB A versus LAB B and LAB C.

Inspection of loadings of pos mode data showed that components from the CDIM such as glucose, amino acids (leucine + isoleucine, (Leu+Ile); phenylalanine (Phe), tryptophane (Trp), tyrosine (Tyr)),...
adenosine (AR) and adenine (A) were positively correlated with principal component 1 (PC1). Accordingly, PC1 correlated negatively with nutrient consumption.

For ESI neg data, PC1 loadings correlated positively with formation of 2-hydroxy acid metabolites, e.g. lactic acid (La), 2-hydroxy-3-phenylpropanoic acid (OH-Phe-Pr), 2-hydroxy (4-hydroxyphenyl)propanoic acid (OH-(OH-Phe)-Pr) and 2-hydroxy-4-methylpropanoic acid (OH-Me-Pr). Consumption of glucose (Glc), amino acids (Phe,Tyr) and the intermediate inosine (IR) correlated negatively with PC1.

According to the highest loadings in PC2 in positive mode, the three LAB strains differed by a preference for metabolizing aromatic (Phe, Tyr, Trp) or branched chain (Leu + Ile) amino acids. Corresponding to this, LAB C produced relatively higher amounts of branched 2-hydroxy acids (seen in negative mode), whereas the LAB B had relatively higher amounts of the corresponding aromatic catabolism 2-hydroxy acids products.

**Exploring the Correlation between Exometabolite Profiles and Mold Growth**

Exploratory multivariate data analysis of the two analytical modes demonstrated that the exometabolome contained information to differentiate the *Lb. paracasei* CFs, both in terms of nutrient consumption and formation of metabolites. The two indicator molds responded differently to the exometabolome of the three LAB strains. The two sets of data (ESI pos and neg) were merged into one X-data block to obtain a more comprehensive description of the differentiation. Low molecular weight organic acids like formic, acetic, propionic, butanoic acid, were analyzed by targeted GC/FID analysis. All these analytes were either below limit of quantification or not detected (table 5) and were not included in the X-data block.

The merged X block was group-scaled (i.e. variance scaled to equal sum-of-square weighing) and mean centered. The Y-block data was mean centered. Partial least squares regression (PLSR) was performed with the mold growth for day 3 for each of the two indicator organisms versus the augmented X. The characteristics of the resulting PLSR models as well as loading and scores plots are listed in supplementary material S3 (see Figs 1H.S3a and b). For both organisms, the PLSR models of all days showed correlation between growth of the indicator organism and exometabolomic profile. Similar correlation was observed for both organisms for mold growth at days 4 and 5 as well. All following observations below are based on PLSR models for day 3 growth of each of the two indicator molds (DCS 302 and DC1 541).
According to the loadings for latent variable 1 in supplementary material, high mold growth, correlated to relatively high amounts of primarily glucose (as several adducts), phenylalanine, leucine + isoleucine and adenosine. Reduced mold growth was most strongly correlated to depletion of these nutrients. The residual amount of each of the highest ranking nutrients was estimated as the sum of relative responses of all the compounds’ adduct and in-source fragment features and are illustrated in Figure 1H.3.

None of the observed nutrients were depleted, except for glucose and glutamine which were almost depleted in LAB C ferments. For all ferments all other measured nutrients remained at more than 50% of the content observed in the CDIM.

Besides consumption of nutrients, formation of metabolites correlated positively with reduced mold growth. Among, the major metabolites were 2-hydroxy-4-methylpentanoic acid (OH-Me-Pe), 2-hydroxy-3-phenylpropanoic acid (OH-Phe-Pr) and 2-hydroxy-3-(4-hydroxyphenyl)propanoic acid (OH-(OH-Phe)-Pr) (table 1H.4).

The variables mostly influencing the overall model were identified by calculation and inspection of variable importance in projection, or VIP scores [21]. For both mold models, the variables were grouped according to a positive or negative correlation to mold growth and within the groups ranked according to VIP scores and listed with the univariate Pearson correlation towards mold growth (Table 1H.7).
Table 1H.7 Features in *Lb. paracasei* CF which correlated positively (top) and negatively (below) in latent variable 1 with mold growth and sorted according to VIP score (decreasing from top to bottom)

<table>
<thead>
<tr>
<th>Observed m/z</th>
<th>Ret. time [min]</th>
<th>ESI Polarity</th>
<th>Ion/adduct</th>
<th>Assignment a</th>
<th>Abbreviation</th>
<th>Exact m/z</th>
<th>Error [ppm]</th>
<th>Pearson correlation coefficient (R) b</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Features positively correlated with mold growth</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>203.0526</td>
<td>1.03</td>
<td>Pos</td>
<td>[M+Na]^+</td>
<td>Glucose</td>
<td>Gic</td>
<td>203.0526</td>
<td>0.2</td>
<td>0.98</td>
</tr>
<tr>
<td>166.0864</td>
<td>4.02</td>
<td>Pos</td>
<td>[M+H]^+</td>
<td>Phenylalanine</td>
<td>Phe</td>
<td>166.0863</td>
<td>0.6</td>
<td>0.72</td>
</tr>
<tr>
<td>132.1020</td>
<td>3.57</td>
<td>Pos</td>
<td>[M+H]^+</td>
<td>Leucine + Isoleucine</td>
<td>Leu + Ile</td>
<td>132.1019</td>
<td>0.5</td>
<td>0.95</td>
</tr>
<tr>
<td>120.0808</td>
<td>4.02</td>
<td>Pos</td>
<td>[M-H-COOH+H]^+</td>
<td>Phe in-source fragment</td>
<td>Phe^*</td>
<td>120.0808</td>
<td>0.5</td>
<td>0.55</td>
</tr>
<tr>
<td>268.1041</td>
<td>3.56</td>
<td>Pos</td>
<td>[M+H]^+</td>
<td>Adenosine</td>
<td>AR</td>
<td>268.1040</td>
<td>0.2</td>
<td>0.87</td>
</tr>
<tr>
<td>183.0917</td>
<td>5.76</td>
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<td>[M+H]^+</td>
<td>Unk-42; C12H10N2</td>
<td>183.09@5.76</td>
<td>-</td>
<td>-</td>
<td>0.87</td>
</tr>
<tr>
<td>137.0459</td>
<td>3.61</td>
<td>Pos</td>
<td>[M-C6H4O2+H]^+</td>
<td>Inosine in-source fragment</td>
<td>IR^*</td>
<td>137.0458</td>
<td>0.6</td>
<td>0.94</td>
</tr>
<tr>
<td>198.0972</td>
<td>1.03</td>
<td>Pos</td>
<td>[M+NH4]^+</td>
<td>Glucose</td>
<td>Gic</td>
<td>198.0972</td>
<td>0.1</td>
<td>0.96</td>
</tr>
<tr>
<td>188.0707</td>
<td>4.64</td>
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<td>[M-NH3+H]^+</td>
<td>Trp in-source fragment</td>
<td>Trp^*</td>
<td>188.0706</td>
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<td>0.36</td>
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<tr>
<td>205.0973</td>
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<td>Pos</td>
<td>[M+H]^+</td>
<td>Tryptophan</td>
<td>Trp</td>
<td>205.0972</td>
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<td>0.09</td>
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<td>267.0736</td>
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<td>[M-H]</td>
<td>Inosine</td>
<td>IR</td>
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<td>0.3</td>
<td>0.97</td>
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<tr>
<td>150.0584</td>
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<td>Pos</td>
<td>[M+H]^+</td>
<td>Methionine</td>
<td>Met</td>
<td>150.0583</td>
<td>0.5</td>
<td>0.80</td>
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<tr>
<td>136.0618</td>
<td>1.94</td>
<td>Pos</td>
<td>[M+H]^+</td>
<td>Adenine</td>
<td>A</td>
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<td>0.21</td>
</tr>
<tr>
<td>180.0867</td>
<td>1.04</td>
<td>Pos</td>
<td>[M-H2O+NH4]^+</td>
<td>Glucose in-source fragment</td>
<td>Glc^*</td>
<td>180.0867</td>
<td>0.2</td>
<td>0.95</td>
</tr>
<tr>
<td>383.1159</td>
<td>1.03</td>
<td>Pos</td>
<td>[2M+Na]^+</td>
<td>Glucose</td>
<td>Gic</td>
<td>383.1160</td>
<td>-0.2</td>
<td>0.97</td>
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<td>Pos</td>
<td>-</td>
<td>Unk-47</td>
<td>231.11@5.39</td>
<td>231.1128</td>
<td>0.0</td>
<td>0.88</td>
</tr>
<tr>
<td>269.0882</td>
<td>3.61</td>
<td>Pos</td>
<td>[M+H]^+</td>
<td>Inosine</td>
<td>IR</td>
<td>269.0880</td>
<td>0.6</td>
<td>0.94</td>
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<tr>
<td>182.0812</td>
<td>3.55</td>
<td>Pos</td>
<td>[M+H]^+</td>
<td>Tyrosine</td>
<td>Tyr</td>
<td>182.0812</td>
<td>0.4</td>
<td>0.32</td>
</tr>
<tr>
<td>118.0863</td>
<td>1.66</td>
<td>Pos</td>
<td>[M+H]^+</td>
<td>Valine</td>
<td>Val</td>
<td>118.0863</td>
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<td>0.91</td>
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<tr>
<td><strong>Features negatively correlated with mold growth</strong></td>
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<td></td>
<td></td>
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<td></td>
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<tr>
<td>131.0714</td>
<td>5.90</td>
<td>Neg</td>
<td>[M-H]</td>
<td>2-hydroxy-Y^-methylpentanoic acid</td>
<td>OH-Me-Pe</td>
<td>131.0714</td>
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<td>-0.92</td>
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<tr>
<td>165.0558</td>
<td>6.39</td>
<td>Neg</td>
<td>[M-H]^+</td>
<td>2-hydroxy-3-phenylpropanoic acid</td>
<td>OH-Phe-Pr</td>
<td>165.0557</td>
<td>0.5</td>
<td>-0.62</td>
</tr>
<tr>
<td>PPM</td>
<td>R</td>
<td>Ions</td>
<td>Monoisotopic Mass (Da)</td>
<td>Molar Mass (Da)</td>
<td>Correlation between mold growth and feature response</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>-----</td>
<td>---</td>
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<td>---------------------------------------------------</td>
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<tr>
<td>181.0507</td>
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<td>Neg</td>
<td>[M-H]</td>
<td>2-hydroxy-3-(4-hydroxyphenyl)propanoic acid</td>
<td>OH-(OH-Phe)-Pr</td>
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<tr>
<td>169.0761</td>
<td>5.47</td>
<td>Pos</td>
<td>[M+H]+</td>
<td>Unk-28; C11H8N2</td>
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<tr>
<td>251.0773</td>
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<td>[M-H]</td>
<td>Unk-981</td>
<td>251.08@2.00</td>
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<tr>
<td>117.0557</td>
<td>4.67</td>
<td>Neg</td>
<td>[M-H]</td>
<td>2-hydroxy-3-methylbutanoic acid</td>
<td>OH-Me-Bu</td>
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<tr>
<td>162.0761</td>
<td>1.89</td>
<td>Pos</td>
<td>[M+H]+</td>
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<td>89.0243</td>
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<td>Neg</td>
<td>[M-H]</td>
<td>Lactic acid</td>
<td>La</td>
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<tr>
<td>204.0667</td>
<td>6.62</td>
<td>Neg</td>
<td>[M-H]</td>
<td>2-hydroxy-3-(1H-indol-3-yl)propanoic acid</td>
<td>OH-Ind-Pr</td>
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<tr>
<td>217.0830</td>
<td>2.47</td>
<td>Neg</td>
<td>[M-H]</td>
<td>Unk-991; C6H14N2O5</td>
<td>217.08@2.47</td>
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<td>5.22</td>
<td>Pos</td>
<td>[M+H]+</td>
<td>Unk-66; C12H12N2O2</td>
<td>217.10@5.22</td>
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<tr>
<td>147.0452</td>
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<td>Neg</td>
<td>[M-H2O-H]+</td>
<td>In-source fragment of PLA</td>
<td>OH-Phe-Pr*</td>
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<tr>
<td>137.0458</td>
<td>2.79</td>
<td>Pos</td>
<td>[M+H]+</td>
<td>Hypoxanthine</td>
<td>Hx</td>
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<tr>
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<td>Pos</td>
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<td>Unk-97</td>
<td>130.09@1.84</td>
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<tr>
<td>219.0976</td>
<td>2.47</td>
<td>Pos</td>
<td>[M+H]+</td>
<td>Unk-40 = Unk-991</td>
<td>219.10@2.47</td>
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<tr>
<td>219.1339</td>
<td>1.71</td>
<td>Pos</td>
<td>[M+H]+</td>
<td>Unk-85; C9H18N2O4</td>
<td>219.13@1.71</td>
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<td></td>
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<td>Unk-1004</td>
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<td>149.0279</td>
<td>4.72</td>
<td>Neg</td>
<td>[M-H]</td>
<td>2-hydroxy-4-(methylthio)butanoic acid</td>
<td>OH-MeS-Bu</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a) Unk = Unknown identified with tentative elemental composition where possible
b) Correlation between mold growth and feature response
c) Y = 4 or 3, i.e. 2-hydroxy-4-methylpentanoic acid or 2-hydroxy-3-methylpentanoic acid
Minimal inhibition concentrations
The contents of selected carboxylic acids were quantified by targeted LC/MS and GC/FID resulted in concentrations listed in Table 1H.8.

Chemical inhibition studies were performed with the 2-hydroxy acids (number 1-6 in table 5 below) individually as presented in supplementary material S4. Minimal inhibition concentration (MIC 50%) of the individual compounds, estimated as the lowest concentration where the inhibition degree was below 0.5 [9], was in the range of 5 to 10 mg/ml for all six compounds. This MIC 50 value was more than 75 times higher than the concentration produced of the most abundant of the six acids (OH-Me-Pe at 65 mg/l).

Table 1H.8 Quantification of produced and identified metabolites with highest VIP scores in mold growth models (average ± stdev, n=5) and minimal inhibitory concentration for 50% inhibition (MIC 50)

<table>
<thead>
<tr>
<th>Compound (abbreviation; number)</th>
<th>L. paracasei strain</th>
<th>LAB A mg/l</th>
<th>LAB B mg/l</th>
<th>LAB C mg/l</th>
<th>Limit of detection mg/l</th>
<th>MIC 50 (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-hydroxy-Y3-methyl pentanoic acid (OH-Me-Pe; #1)</td>
<td>12.3 ± 0.2</td>
<td>24.5 ± 0.5</td>
<td>65 ± 6</td>
<td>0.3</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>2-hydroxy-3-phenylpropanoic acid (OH-Phe-Pr; #2)</td>
<td>&lt;2</td>
<td>21.1 ± 0.9</td>
<td>11.6 ± 1.0</td>
<td>0.8</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>2-hydroxy-(4-hydroxyphenyl)propanoic acid (OH-(OH-Phe)-Pr; #3)</td>
<td>&lt;1</td>
<td>15.8 ± 0.7</td>
<td>6.5 ± 0.4</td>
<td>0.4</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>2-hydroxy-3-methylbutanoic acid (OH-Me-Bu; #5)</td>
<td>5.6 ± 0.1</td>
<td>5.3 ± 0.3</td>
<td>23.4 ± 2.2</td>
<td>0.7</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Lactic acidb (La)</td>
<td>9.6 x 10³ ± 0.6 x 10³</td>
<td>15.6 x 10³ ± 0.5 x 10³</td>
<td>16.8 x 10³ ± 0.4 x 10³</td>
<td>1 x 10³</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>2-hydroxy-3-(1H-indol-3-yl)propanoic acid (OH-Ind-Pr; #4)</td>
<td>&lt;3</td>
<td>4.4 ± 0.2</td>
<td>&lt;3</td>
<td>0.9</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>2-hydroxy-4-(methylthio)butanoic acid (OH-MeS-Bu; #6)</td>
<td>1.2 ± 0.03</td>
<td>4.4 ± 0.2</td>
<td>2.7 ± 0.2</td>
<td>0.2</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Formic acidb (Fo)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>10</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Acetic acidb (Ac)</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>3</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>Propanoic acidb (Pr)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>1</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Butanoic acidb (Bu)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>1</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>
a) Y = 4 or 3, i.e. 2-hydroxy-4-methylpentanoic acid or 2-hydroxy-3-methylpentanoic acid
b) Quantified by GC/FID

ND: Not detected, i.e. below limit of detection
<xx: Detected, but below limit of quantification of xx mg/ml

NA: Not analyzed

Mold Growth on Simulated Ferments
The effect of simulated ferments on mold growth, i.e. without and with the six 2-hydroxy acids (number 1-6) in the concentrations listed in table 1H.5, is illustrated in fig 1H.4. Absence of glucose further reduced mold growth compared to SIMLAB C with acids (data not shown).

Data analysis by multifactor analysis of variance (ANOVA) for mold growth with factors pH (3.8; 4.6; 4.5) and presence/absence of the six 2-hydroxy acids showed both factors to have a significant effect on DCS 1541. For DCS 302, only pH had a significant effect and the presence of the acids did not significantly affect the mold growth. Variance component analysis with the factor acid presence/absence nested within the factor pH demonstrated that pH was the main factor towards both Penicillium strains. DCS 302 appeared to be more sensitive towards pH than DCS 1541 as the variation in pH explained 95% and 78%, respectively. With this approach, the acid presence accounted for 0% and 9% of the variation for DCS 302 and DCS 1541, respectively. The remaining 5 and 12% was unsystematic variation which exceeded the effect of acid presence.

DISCUSSION
Recent studies showed that a major contributor to the antifungal properties of LAB A (Lb. paracasei DGCC 2132) owed to release of diacetyl during the metabolism of live cells. Removal of the cells effectively reduced the diacetyl concentration and consequently reduced the inhibitory effect. After removal of cells, some residual antifungal effect remained in the cell free ferment, indicating other factors contributing to antifungal activity (Aunsbjerg, Honore, Marcussen, Ebrahim, Vogensen, Benfeldt, Skov and Kn0chel: Contribution of diacetyl to the antifungal effect of Lactobacillus paracasei in defined medium and yogurt, submitted for publication).

The purpose of this Example was to demonstrate the use of an untargeted metabolomics approach for investigation of the factors responsible for the antifungal effect observed in CFs of selected Lb. paracasei strains. Fermentation of CDIM by the selected Lb. paracasei strains induced relative changes in the exometabolomes in terms of a) consumption of nutrients and b) formation of metabolites. By removal of cells and hence contribution from diacetyl, the relative growth of molds
on the cell free ferments (CF) would provide the biological response to the combined change in the exometabolomes and hereby assist for assigning the factors that contributes to antifungal effect. Potentially, this approach could serve as a supplement to bio-assay guided fractionation for identifying antifungal metabolites.

The three *Lb. paracasei* strains were selected to display significant variation in effect on mold growth, i.e. inhibitory properties. As expected, mold growth was different on the three types of *Lb. paracasei* CF ferments. However, LAB B and C produced more biomass and acidified to lower pHs than LAB A. All three strains were diacetyl producers, but the inhibition factor from diacetyl was considered to be effectively reduced by removing cells by centrifugation and filtration (Aunsbjerg, Honore, Marcussen, Ebrahimi, Vogensen, Benfeldt, Skov and Kn0chel: Contribution of volatiles to the antifungal effect of *Lactobacillus paracasei* in defined medium and yogurt, submitted for publication).

Mold growth was assessed by recording and analyzing multispectral images. Spores of *Penicilium* are normally white in the initial growth phase. As the mold matures the color of the spores change into green-grey or blue-green, and the development is dependent e.g. on the composition of the medium [22]. By the use of multispectral images, the approach allows for precise and objective description of the growth of *Penicilium* spp. by measuring the white, green segments and the total area of the mold growth (sum of white and green segments) (Ebrahimi, van den Berg, Nielsen, Honore, Benfeldt, Jensen and Engelsen: Quantitative determination of mold growth and inhibition by multispectral imaging, submitted).

Visually observed differences in mold growth during incubation were best described by the development of total (or green) amount of pixels as the number of white pixels remained constant.

The LC/MS-based metabolic footprinting of the CF was designed to include a wide range of nutrients and metabolites. However, the use of the reversed phase protocol excluded compounds more hydrophilic than e.g. glucose and lactic acid. Furthermore, the LC/MS footprint did not include low molecular short chain fatty acids like formic, acetic, propionic and butanoic acids. According to literature, these compounds contribute to the antifungal properties of lactic acid bacteria (e.g. [9, 10]) and were included in the study via GC/FID analysis. All four acids were either not detected or below a limit of quantification, which was more than 300 times below MIC values (e.g. MIC of acetic acid at pH 4.0 [9]: 3 g/L). Hence, these were not included in the multivariate data analyses.
In spite of these limitations, the three *Lb. paracasei* strains could readily be classified by PCA. This classification was based on both the relative consumption of nutrients and formation of metabolites.

The sole purpose of doing PLSR was to identify compounds in the exometabolome which correlate with mold growth. The very limited number of *Lb. paracasei* strains did not allow the model to be used for any prediction of mold growth. Although the two indicator molds responded slightly different to the three *Lb. paracasei* CFs, their respective PLSR models showed practically similar ranking of the variables.

In terms of the consumption of nutrients, glucose ranked highest and was together with glutamine the only medium components almost fully consumed (by LAB C). Other nutrients remained at 50% or more of the initial content. The metabolites which had the highest correlation with decreased mold growth was lactic acid (primarily fermentation product of glucose), together with additional six 2-hydroxy carboxylic acids and several unidentified compounds.

Two metabolites, probably intermediates from metabolism of nucleotides [23], namely inosine hypoxanthine also correlated with mold growth. However, the two compounds were not considered relevant factors for reduced mold growth based on the absence of anti-mold effect previously reported for other nucleosides [1].

Based on the PLSR model and GC/FID data it was hypothesized, that the minimal mold growth should be due to primarily three factors, namely (complete) consumption of glucose, acidification by lactic acid and formation of six known metabolites.

Mold growth on simulated fermentations of the three *Lb. paracasei* was performed to assess the effect of the three factors and hereby validate the hypothesis outlined via the PLSR model. The relative growth of both indicator molds on the simulated fermentations correlated with the growth on the actual ferments. The effect of lactic acid appeared as the main factor for both indicator strains and was responsible for the major part of the observed variation. Absence of glucose served as an additional factor for reduced mold growth. Only for DCS 1541 the addition of the produced 2-hydroxy acids had a minor but significant effect on mold growth. It is interesting to notice that an effect was observed at concentrations of the six acids used. This could indicate a combined effect towards DC 1541.
To the inventors knowledge, indolelactic acid, 2-hydroxy-(4-methylthio)butanoic acid and 2-hydroxy-3-methylbutanoic acid have previously not been reported as antifungal compounds produced by *Lb. paracasei* strains. MIC 50 values obtained during this study were in accordance with the previously reported 2-hydroxy acids from LAB (2-hydroxy-4-methyl-pentanoic acid - MIC 10 g/L [24], phenyllactic acid - MIC 4 g/L [25], 4-hydroxyphenyllactic acid - MIC 5 g/L [1]).

The six 2-hydroxy acids were recognized as metabolites from lactic acid bacteria catabolism of amino acids [18, 26]. Their structural similarity with amino acids (Leu, Phe, Tyr, Trp, Met, Val) suggested that the metabolites were formed via the transamination route via a keto acid, which is reduced by a hydroxyacid dehydrogenase (Fig. 1H.5). This is supported by the loadings observed where e.g. 2-hydroxy-3-phenylpropanoic acid was inversely correlated with the precursor Phe Fig 1H.2.

Even though the screening method favored these compounds, it was striking that transaminated and reduced metabolites were so abundant in ESI neg mode. The most obvious explanation could be a significantly higher MS response of these components compared to metabolites from the alternative route in the transamination, where the keto-acids are decarboxylated and reduced/oxidized. Products from this pathway would be aldehydes, alcohols and carboxylic acids with a Cn-1 carbon chain. Aldehydes and alcohols are expected to have lower responses in ESI neg mode than corresponding carboxylic acids. These metabolites could have been detected by a more sensitive method like headspace sampling followed by GC/MS [18]. The headspaces of the fermentiones were analyzed during fermentation by SPME-GC/MS to test for decarboxylated metabolites. For LAB A, B and C the main observed components were diacetyl and acetoin and the method was not able to detect any components related to the decarboxylation metabolic pathway outlined in fig. 1H.5.

However, detecting the 2-hydroxy acid metabolites is in accordance with presence of the genes for both D- and L-hydroxy acid dehydrogenases (HycDH) in *Lactobacilli* (and especially for *Lb. easel*) as described by Liu [27]. It was interesting to notice that none of the corresponding keto acids were detected being the precursors for the hydroxy acids.

Although the used exometabolomic approach is simplistic, it demonstrates the potential embedded in including the combinations of compounds. Hereby, it holds promise being a valuable supplement to e.g. bioassay guided fractionation for finding novel compounds and factors responsible for antimicrobial effect.
CONCLUSION
Exometabolomic footprints of Lb. paracasei strains were correlated to mold growth to assist in identifying the factors responsible for the antifungal properties of the bacteria. For the selected mold growth test system, both nutrient consumption and metabolite formation correlated with the inhibition observed. The study demonstrated metabolic footprinting as a valuable supplement to bioassay guided fractionation for investigation of antifungal properties of bacterial cultures.
Additionally, the strategy enabled the detection and assignment of three previously identified and three novel antifungal compounds and indicated potential precursors for the metabolites.

Supplemental information S3: Table 1

Table 1 Characteristics of the PLSR of augmented merged X against growth of the two indicator molds, day 3

<table>
<thead>
<tr>
<th></th>
<th>PLSR on Mold growth of</th>
<th>DGCC 302</th>
<th>DGCC1541</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biological samples included</td>
<td>9</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Number of X-block variables</td>
<td>1119</td>
<td>1119</td>
<td></td>
</tr>
<tr>
<td>Number of Latent Variables</td>
<td>4</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>%Variation explained, X-block, PC1</td>
<td>66.67</td>
<td>68.12</td>
<td></td>
</tr>
<tr>
<td>PC2</td>
<td>29.29</td>
<td>27.85</td>
<td></td>
</tr>
<tr>
<td>PC3</td>
<td>1.54</td>
<td>0.92</td>
<td></td>
</tr>
<tr>
<td>PC4</td>
<td>1.17</td>
<td>1.69</td>
<td>0.45</td>
</tr>
<tr>
<td>%Variation explained, Y-block, PC1</td>
<td>81.41</td>
<td>94.78</td>
<td></td>
</tr>
<tr>
<td>PC2</td>
<td>7.11</td>
<td>1.59</td>
<td></td>
</tr>
<tr>
<td>PC3</td>
<td>7.94</td>
<td>3.07</td>
<td></td>
</tr>
<tr>
<td>PC4</td>
<td>2.44</td>
<td>0.31</td>
<td>0.18</td>
</tr>
<tr>
<td>Root Mean Square Error of Calibration</td>
<td>85</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Root Mean Square Error of Cross Validation</td>
<td>439</td>
<td>281</td>
<td></td>
</tr>
<tr>
<td>Correlation coefficient, R²</td>
<td>0.784</td>
<td>0.934</td>
<td></td>
</tr>
<tr>
<td>Calibration Bias</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>CV bias</td>
<td>-51</td>
<td>-1.9</td>
<td></td>
</tr>
</tbody>
</table>

Supplemental information/material S4: Table 1 - See Table 1C.2 of Example 1C.

REFERENCES for Example 1H


Example 1J - Quantitative determination of mold growth and inhibition by multispectral imaging

List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDIM</td>
<td>Chemically Defined Interaction Medium</td>
</tr>
<tr>
<td>GUI</td>
<td>Graphical User Interface</td>
</tr>
</tbody>
</table>

Quantifying mold growth is of great relevance and interest in microbiology. However, predictive modeling of filamentous fungal growth has been hampered by the lack of an appropriate and accurate method for quantification. An adequate, rapid and objective method will allow studying the effect of many different parameters and conditions on mold growth patterns and can thus provide valuable insight and knowledge. This Example outlines a new approach for quantifying mold growth by providing an accurate tool for measuring different segments of mold colonies. The method is based on clustering multispectral images by \( k \)-means, an unsupervised and simple clustering algorithm. In order to demonstrate the efficiency of the new approach, three different sample sets were analyzed by the developed method, with the objective of quantifying mold growth and size of the colony segments of \textit{Penicilium} mold. The results verify the ability of the proposed method to quantify mold growth and colony composition (relative size of the white and green segments) accurately. This provides a robust measure for interpreting inhibition activity against mold in different samples and makes a quantitative comparison possible. Among the virtues of the method are: 1) the ability to quantify very small differences in the size of colonies which cannot be easily discriminated by visual inspection, 2) the ability to quantify mold growth on transparent as well as on opaque media (e.g. milk), and 3) no prior assumptions for the shape and multiplicity of colonies. The accuracy and non-destructive characteristic of the method allow dynamic quantification of mold growth which can be very valuable in predictive microbiology and in studies related to biopreservation of food products.

1. Introduction

In food microbiology, it is of great relevance and interest to quantify mold growth and investigate how different parameters influence the process - for instance, in studies related to biopreservation of food products, where safe bacteria are used to inhibit growth of disease-causing microorganisms (Chaillou et al., 2005). However, predictive modeling of filamentous fungal growth has been hindered by the lack of adequate quantitative methods (Marin et al., 2005; Marin et al., 2008). A number of approaches have been reported and used for this purpose, including colony forming units (CFU) counts, total ergosterol content and colony diameter (Marin et al., 2005). CFU is one of the most frequently used methods for quantifying mold growth, but it suffers from serious
drawbacks. It usually reflects spore numbers rather than biomass and is, in general, a poor
indicator of the extent of fungal growth. CFU also appears to correlate poorly with other parameters
such as ergosterol content (Taniwaki et al., 2006) which is the second most commonly used
method to quantify mold. Ergosterol is the dominant sterol in most fungi, and its concentration
accounts for the total fungal population in a food sample (Taniwaki et al., 2006). Although
ergosterol has shown good performance as a fungal growth indicator for different species (Marin et
al., 2008), it is not possible to determine ergosterol concentration accurately for very small colonies
(e.g. a colony as small as 2 mm in diameter). By far, the simplest method to assess mold growth is
measuring the colony diameter (or area). As molds often grow in the form of surface colonies,
colony diameters can be measured on petri dishes over time and converted into growth curves
(Taniwaki et al., 2006). Colony diameter measurements show higher repeatability and sensitivity
compared to ergosterol measurements (Marin et al., 2006), and diameters of very small colonies -
for which ergosterol content cannot be measured accurately - can be easily determined. Although
colony diameter does not take colony density and volume into account, it is the most suitable
measure of the fungal biomass in solid substrates (Garcia et al., 2010). Good correlation has been
reported between ergosterol content and colony diameter (Marin et al., 2006). Colony diameter
and size measurement is also non-destructive, and therefore, saves the sample for further analysis
or time-series studies. In General, colony diameter is measured manually (Wang, et al. 2012), and
sometimes just a visual inspection of the colonies is used to estimate mold growth and grade
inhibition (Magnusson and Schnurer 2001). For manual measurement of colony area, the routine
practice is to measure the diameters of the mold in the two main perpendicular directions and
estimate the area or to overlay tracing paper on the mold colony, trace the shape, and then overlay
the tracing paper on graph paper and count the squares. These procedures obviously lack
accuracy and precision and they can be even less reliable when colonies have not grown in well-
shaped circular forms. In addition, they can disturb the mold, and spread the spores around which
will bias the results by increasing the apparent growth. Moreover, if the investigated molds are
toxic, manual measurement of the colonies can pose potential health risks to the analyst.

This Example proposes a new and semi-automated approach for quantifying mold growth based
on the colony size or area, using the unsupervised /k/-means clustering of multispectral images,
recorded in the ultraviolet, visual and near-infrared regions. Multispectral imaging combines
spectroscopy and imaging, and thus provides both spectral and spatial information on the samples,
which in this Example are mold colonies grown on different media in standard petri dishes. The k-
means clustering algorithm is a simple procedure which was employed to subdivide multispectral
images of the mold colonies on petri dishes, and quantify different segments of the mold colonies.
In order to test and demonstrate the efficiency of the new approach, three different sample sets were analyzed with the objective of quantifying white and green segments of *PeniciHium* mold colonies. As white and green segments of the colonies relate to different stages of sporulation, their individual quantification can be informative. The first set of the analyzed samples was inhibition assays for indicator fungi spotted on the cell-free ferments of antifungal bacterial cultures in a chemically defined interaction medium (CDIM), which had a transparent background. The second set of samples was inhibition assays for indicator fungi spotted on the acidified uninoculated CDIM samples with different pH values. The third set of samples consisted of inhibition assays for indicator fungi spotted on the ferments of the microbial strains on a milk-based medium, which had an opaque background. The new method, called 'PCluster', performs the analysis of the images in a semi-automatic way and is distributed as a freely available MATLAB Graphical User Interface (GUI). The results verify the ability of the proposed strategy to quantify mold growth and colony composition in response to an inhibitor challenge, both on transparent and opaque media. By colony composition, the inventors refer to the relative area (size) of the white and green segments of a colony. For example, if there are two colonies having the same total area, it can be deducted that the one with the bigger size of the green segment, is more advanced in growth. This is one of the arguments which make separate quantification of the different segments of the mold colony advantageous.

2. Materials and methods

2.1. Developed approach for quantifying mold growth

The *k*-means clustering algorithm was used to subdivide multispectral images of the mold colonies on petri dishes and quantify different segments of the mold colonies. A graphical illustration of the procedure is presented in Figure 1J.1. Images were acquired using the Videometerl_ab2 instrument (Videometer A/S, Hørsholm, Denmark). In these images, each pixel is associated with a spectrum and can be considered as an object in a *w*-dimensional space, where *w* is number of the wavelengths. Each image data cube is unfolded into a matrix, where the *n* rows are the pixels and the *w* columns are the wavelengths (*n* ≫ *w*, see Fig. 1J.1). Then, the *k*-means algorithm is used to cluster the pixels in this unfolded matrix, using the information from all the spectral bands. The concept is the same as clustering objects in e.g. a three-dimensional Cartesian coordinates, just expanded to a *w*-dimensional space. The final outputs of the analysis are quantification of different segments of the mold colonies in pixel-counts and the corresponding average spectrum for each segment. For instance, for the *PeniciHium* molds that were used in this Example, mold colonies consist of white and green segments. Results of the analysis are thus number of the white and
green pixels in the mold colony and their average spectra (see Fig. 1J.1). This allows quantifying
the mold growth as well as the composition of the colony.

To perform the clustering and analysis of the images in a semi-automated way, a Graphical User
Interface (GUI) has been developed using MATLAB 2012b (MathWorks, Inc., Natick, MA, USA).
The layout of the GUI, which is called 'PCluster', is shown in Figure 1J.2. PCluster is specifically
designed for Penicillium molds, for which the colonies are composed of white and green segments.
However, the method and the explained concept can be used for all types of molds. Multispectral
images are first imported into PCluster, and then the user selects a circular region of interest which
will be used for all the images in the imported set. In the /c-means algorithm, the number of clusters,
k, is a user defined input. In PCluster, images are clustered from 3 to 6 groups and the results are
shown as color-coded (so-called false negative) image objects which show membership of the
pixels in the clusters (see Fig. 1J.2). Then, based on the graphical output, the optimum number of
clustering and the meaningful clusters, k, are selected by the user. Meaningful clusters are the
ones which show the colonies segments, and the optimum number of groups is the one which
allows clustering different parts of the mold colony properly. For instance, in Fig. 1J.2, partitioning
the pixels into 4 clusters is enough to segment the white and green parts of the mold colony and
separate them from the background. Selecting more than the optimum number of clusters will
subdivide the colony segments further and will impose some errors on the quantification results,
since only two colony segments can be chosen. The user can make a specific interpretation to the
clusters, based on the color-coding of the pixels and select the meaningful clusters by ticking the
corresponding check boxes (see Fig. 1J.2). Although it may not seem so at the first sight, the
procedure is quite simple and will come natural to the user in a short while.

This method is more accurate than the manual measurement of mold colony size. Figure 1J.3
shows two images of mold colonies and also the clustered images, which allow quantification of
the different segments of the colonies as different clusters of pixels. It is obvious that manual
measurement of these colonies in perpendicular directions and calculating the area would not
provide the true size of the colonies, as the colonies do not grow quite symmetrically.

2.2. Microorganisms and culture conditions
The bacteria and molds which were used in this Example are listed in Table 1J.1. All bacterial
strains were freeze dried and kept at -18 °C until use. Indicator molds were stored at -80 °C, in 20%
glycerol and water, containing 0.1% Tween 80 (Merck).
Table 1J.1 - Bacteria, commercial cultures and fungi used in the study and their incubation temperatures.

<table>
<thead>
<tr>
<th>Microbial strain</th>
<th>Incubation temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CDIM</td>
</tr>
<tr>
<td><strong>Antifungal bacteria</strong></td>
<td></td>
</tr>
<tr>
<td><em>Lactobacillus paracasei</em> DGCC 2132</td>
<td>37</td>
</tr>
<tr>
<td><em>Lactobacillus paracasei</em> DGCC 11287</td>
<td>37</td>
</tr>
<tr>
<td><strong>Commercial cultures</strong></td>
<td></td>
</tr>
<tr>
<td>YO-MI)^410</td>
<td>-</td>
</tr>
<tr>
<td>HolDBACI YM-C</td>
<td>-</td>
</tr>
<tr>
<td><strong>Indicator fungi</strong></td>
<td></td>
</tr>
<tr>
<td><em>Penicillium</em> sp. DCS 1541</td>
<td>25</td>
</tr>
<tr>
<td><em>Penicillium solitum</em> DCS 302</td>
<td>25</td>
</tr>
<tr>
<td><em>Penicillium</em> glabrum DCS 305</td>
<td>25</td>
</tr>
</tbody>
</table>

2.3. Ferments of chemically defined medium

Chemically defined interaction medium (CDIM) was prepared as described by Aunsbjerg et al. (2013-submitted for publication). In addition, an enhanced medium (CDIM^a) was prepared by adding an extra agent to the standard medium (proprietary information, DuPont Nutrition Bioscience ApS, Brabrand, Denmark). Both media were inoculated with 10^7 CFU/mL of *Lactobacillus paracasei* DGCC 2132 (LAB A) or *Lactobacillus paracasei* DGCC 11287 (LAB B) and incubated for 22 hours at 37 °C. All batches were made in triplicate. An overview of the batches and the average pH values are presented in Table 1J.2. After fermentation, all the batches were centrifuged (5,000 g, 15 min at 5 °C), followed by a filtration step through a 0.45 µm filter (Frisenette ApS, Knebel, Denmark). Two plates of cell-free ferments were prepared for each replicate, by mixing the extracts with agar (1%) and pouring into petri dishes.

Table 1J.2 - An overview of the batches from the ferments of the chemically defined medium (CDIM). 'Lb.' is the abbreviation for 'Lactobacillus'.

<table>
<thead>
<tr>
<th>Batches</th>
<th>Description</th>
<th>Average pH Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAB A</td>
<td><em>Lb. paracasei</em> DGCC 2132 in CDIM</td>
<td>4.54</td>
</tr>
<tr>
<td>LAB A*</td>
<td><em>Lb. paracasei</em> DGCC 2132 in CDIM^a</td>
<td>4.33</td>
</tr>
<tr>
<td>LAB B</td>
<td><em>Lb. paracasei</em> DGCC 11287 in CDIM</td>
<td>5.72</td>
</tr>
<tr>
<td>LAB B*</td>
<td><em>Lb. paracasei</em> DGCC 11287 in CDIM^a</td>
<td>4.46</td>
</tr>
</tbody>
</table>

2.4. Acidified un-inoculated chemically defined medium samples

To investigate the influence of acidification with lactic acid on mold growth inhibition, un-inoculated samples were prepared by titrating CDIM with 80% DL-Lactic acid to pH values of 4.0, 4.5, 5.0, 5.5,
6.0 and 6.5 in the 6 samples. Subsequently, two plates of each sample were prepared by mixing with agar (1%) and pouring into petri dishes.

2.5. Ferments of milk-based medium

Skim milk powder (Lactalis, Laval, France) was dissolved in distilled water (10% solution) and heat-treated at 90 °C for 10 minutes. To prepare reference (control) batches, milk solutions were inoculated with 20 DCU (Internal dosing unit) of YO-MIX® 410 starter culture. For HOLDBAC® YM-C (HB) batches, in addition to 20 DCU of the starter culture, milk solutions were inoculated with 20 DCU of HOLDBAC® YM-C, which is an antifungal culture. Control batches were included to provide a reference to compare the HB batches with and investigate how the antifungal culture can enhance inhibition. Prior to inoculation, the pH of the heat-treated milk was approximately 6.5. After inoculation, both the control and HB batches were fermented to a pH value of approximately 4.6. Four biological replicates of reference and HB were prepared and incubated at 43 °C for 8 hours. Then, for each replicate, six plates of cell-containing ferments were prepared by mixing with agar (1%) and pouring into petri dishes.

2.6. Mold inhibition test

Inhibition tests were performed using Penicillium sp. DCS 1541 and Penicillium solitum DCS 302 indicator fungi for CDIM batches, and Penicillium sp. DCS 1541 and Penicillium glabrum DCS 305 for the ferments of milk-based medium (see Table. 1J.1). In all the three sample sets, half of the prepared sample plates were used for each of the tested indicator fungi. Plates of CDIM and milk ferments were spotted with 20 µL of 10⁵ spores/mL indicator molds. CDIM plates, both inoculated and un-inoculated batches, were spotted in triplicate, whereas milk plates were spotted with a single spot. Ferments of CDIM, acidified un-inoculated samples and milk plates were incubated at 25 °C for 4, 5 and 6 days, respectively.

2.7. Multispectral image acquisition

A VideometerLab 2 spectral imaging instrument (Videometer A/S, Hørsholm, Denmark) was used to acquire the multispectral images. To record images by this instrument, the sample is placed inside the sphere of the instrument - so-called Ulbricht sphere - where diffused light from light emitting diodes (LEDs) is provided at 18 different wavelengths, ranging from 405 to 970 nm, and a single-channel image is recorded for each wavelength. The lid of the petri dishes was removed prior to image acquisition to avoid reflection. The size of all the acquired images was 2056x2056x18 and the size of each pixel, for the present configuration and instrumental settings, was 45.8 µm x 45.8 µm. For the ferments of CDIM, multispectral images were recorded after 2, 3
and 4 days of incubation of the indicator molds which were used to perform the inhibition tests. For
the acidified samples, multispectral images were recorded after 3, 4 and 5 days of incubation of the
indicator molds. The samples were taken out of the incubator, the images were recorded and then
the samples were returned back to the incubator. Recording the images is quick, taking less than a
minute per sample. For the milk ferments, multispectral images were recorded only once, after 6
days of incubation of the indicator molds. Some representative images (shown here as
conventional RGB) of the CDIM and milk samples after incubation of the indicator molds are shown
in Fig. 1J.4.

2.8. Analysis of multispectral images

The images were analyzed by PCluster, as detailed in Section 2.1. For all the acquired images, the
number of the white and green pixels in the mold colony and their average spectra were calculated.
The analysis time depends on the performance of central processing unit (CPU) of the computer
and also the size of the images. For this Example, the required time was on average a few minutes
per image, using a standard office computer. Unwanted growth on some of the plates, caused by
splashes of the mold solution while spotting (see Fig. 1J.3 and 1J.4), was removed from the
images before quantification, using one of PCluster's built-in options. This allows saving some of
the images while avoiding quantitative errors. PCluster, including the full MATLAB source code, is

3. Results

In this section, the results of analyzing the images from the three designed experiments are
presented and interpreted. The goal was to demonstrate the capability and potentials of the
method in providing a quantitative comparison between different samples, in terms of mold growth
and inhibition. The difference between the inhibition properties of the samples, although briefly
discussed, is not the main interest of this Example and is merely included to verify the performance
of the method and provide examples of the analysis results.

3.1. Ferments of chemically defined medium

For the ferments of the CDIM, the images were analyzed with the aim of quantifying the size of the
green and white segments of the mold colonies as a function of incubation days. The calculated
sizes of the segments (in pixels unit) are shown in Fig. 1J.5a. For all the batches and both tested
molds, after 2 days of incubation, mold colonies are quite similar in size. Results show that at this
stage, the colonies are only composed of white segments; green segments, which indicate a more
advanced stage of the mold growth, have not appeared yet. After 3 days of incubation, mold
colonies grow larger and, as the spores mature, they turn from white to green. According to the colony sizes, the inhibition of Penicillium sp. DCS 1541 is higher in comparison with Penicillium solitum DCS 302. Moreover, the mold colonies grew less on the batches with the enhanced medium (LAB A* and LAB B*) compared to the batches with the standard medium (LAB A and LAB B). Among all the batches and for both of the indicator molds, the lowest inhibition is observed for LAB B. After 4 days of incubation, the growth patterns between the molds are discriminated better and the batches spotted with these molds are separated as two distinct groups in Fig 1J.5a. The average spectra of the green and white segments of the colonies, as shown in Fig 1J.5b, have different patterns and their intensities are clearly different in all the spectral channels, as expected.

3.2. Acidified un-inoculated chemically defined medium samples

For the acidified un-inoculated CDIM, the images were analyzed and the green and white segments of the mold colonies were quantified. The aim was to investigate and compare the growth patterns of the two indicator molds in response to pH variations. For both of the indicator molds, the correlation between the size of the green and white segments (and also the sum of the two segments) with pH of the samples and the growth of the colonies were examined. The best correlation was observed for the green segments and data from the white segments will not be shown or discussed further. Sizes of the green segments of the colonies, as a function of the pH of the samples at different days of the incubation are shown in Fig. 1J.6. For both of the indicator molds, size of the green segments in the colonies naturally increases by days of incubation. For Penicillium solitum DCS 302, growth seems to be inhibited at lower pH values and this trend is observed for all days. By increasing the pH from 4.0 to 5.5, growth increases, whereas by increasing the pH further, the growth does not seem to be influenced. Contrary to Penicillium solitum DCS 302, growth in Penicillium sp. DCS 1541 is not significantly affected by pH. For Penicillium sp. DCS 1541, the image of the sample with pH 6 in day 5 of incubation was not recorded properly and could not be analyzed.

3.3. Ferments of Milk-based medium

For the ferments of milk-based medium, images were analyzed with the aim of quantifying green and white segments of the mold colonies. Results are shown in Fig. 1J.7.

For control (reference) batches, which were only inoculated with the starter culture, mold colonies have grown much larger compared to HB batches, that in addition to the starter culture were inoculated with a culture known to have antifungal properties. On average, considering the total colony size regardless of the composition, Penicillium sp. DCS 1541 colonies on the control
batches are 3 times larger than on the HB batches. For *Penicillium glabrum* DCS 305, colonies are approximately 9 times larger on the control compared to the HB batches. In the control batches, *Penicillium glabrum* DCS 305 has a significantly higher growth rate compared to *Penicillium* sp. DCS 1541. Sizes of the green and white segments of *Penicillium glabrum* DCS 305 colonies are respectively around 5 and 3 times larger, when compared to *Penicillium* sp. DCS 1541. For HB batches, the colonies are only composed of white segments, and the more mature green spores have not appeared, even after 6 days of incubation. Although size of the colonies for the two types of the molds are quite close in HB batches, they still appear as two separate groups in Fig 1J.7a. The average spectra of the green and white segments of the colonies are shown in Fig 1J.7b. As expected, white and green segments of the mold colonies have different spectral patterns and their intensities are also different in all the spectral channels.

4. Discussion

For the ferments of the CDIM, the developed approach - Pcluster - could accurately quantify mold growth, based on the area of the white and green segments of the mold colonies. The areas are presented in pixels unit. Pixel is the building unit of an image and for all of the images in the current Example, the size of each pixel was 45.8 μm × 45.8 μm. As the number of the pixels and their size was constant between the images, pixel could easily be used as a measure of area for a quantitative comparison of mold growth. Converting number of the pixels to the more commonly used units of area, is just a matter of multiplying them by the area of each pixel. Differences between the growth patterns of the two indicator molds, at different days of incubation, could be discriminated. Moreover, the results could show how mold growth was influenced by the change in the medium.

For the acidified CDIM samples, influence of the pH on the growth of the indicator molds could be quantified. The method detected the differences between the two indicator molds in terms of their response to pH variation and their growth patterns. The variation which was created between the samples could be converted into objective measures. This can be very helpful in investigating how different parameters influence mold growth patterns.

The method could also quantify mold growth and size of the white and green segments in the ferments with the milk-based medium. Looking at the RGB images of some of the milk plates, the white edge of the mold colonies is not easily distinguishable on the white milk background (see Fig. 1J.3 and Fig. 1J.4), whereas the proposed method, benefitting from the advantages of multispectral imaging in combination with /-means clustering, could quantify the white sporulating
segments reliably. For the HB batches, the differences in the size of the colonies for the two types of the indicator molds were small, and visual inspection of the plates would not allow deciding which mold was inhibited more efficiently. The results also demonstrated how the mold growth and colony composition (relative size of the segments) differs between the HB and the control batches. The average spectra of the white and green segments have very different spectral patterns in both the CDIM and milk-based samples, and it is because of these different spectral patterns of the colony’s segments that they can be reliably clustered as different groups by the $k$-means algorithm.

The method does not make any assumptions for the shape of the colonies, and can also quantify mold growth on transparent media, like the CDIM used in the first and second sets, as well as on opaque media, like the milk-based medium in the third set. Furthermore, the semi-automated analysis, using PCluster, can discriminate between the molds in a less labor-intensive and a more objective way. Following mold growth with appropriate imaging systems that allow comparative studies over time, and using the developed methodology for quantifying the colonies can be very useful in predictive microbiology and in studying how different parameters affect biopreservation of food products by antifungal bacterial cultures.

**Theory**

*K-means Clustering*

K-means clustering is an unsupervised algorithm that aims to find the best partitioning of $n$ observations (or objects) into $k$ clusters or groups, where $k$ is a number defined by the user. The algorithm starts by randomly selecting $k$ points (objects) as the initial groups’ centroids. Then, the Euclidean distance between all the objects and the centroids are calculated and each object is assigned to the cluster to the centroid of which it is the closest. In the next step, for each cluster, the object which is the most similar to the average of all the objects in the $j$th cluster is defined as the new centroid and objects are clustered again, based on their distance from the new centroids. The process of finding the new centroids and re-clustering the objects is repeated iteratively until the convergence criterion is met. The convergence criterion used in this method is minimizing the Within Cluster Sum of Squares (WCSS), which is the average squared Euclidean distance between the objects and their cluster centroids. This is a measure of how well each centroid represents the group or cluster members. The algorithm has converged when WCSS does not decrease any further with iterations or decreases below a predefined threshold (Mac Queen, 1967; Mohd et al., 2012; Tran et al., 2005). Generally, for each clustering, replicate runs/restarts are performed. Repeats of $k$-means will help to make sure that the algorithm does not converge to local minima. Each one of the replicates begins from a different randomly selected set of initial
centroids and the final solution that /c-means returns is the global minimum which has the lowest WCSS.

**Multispectral Images**

Each multispectral image is a three-dimensional data structure in which two of the dimensions provide spatial information of the sample, and the third dimension represents spectral information for each picture-element (pixel; see Fig. 1J.1). Spectral information is provided for a range of wavelengths, including ultraviolet, visible, and near-infrared which can provide much more information about the samples compared to e.g. the ordinary trichromatic (RGB) images. Multispectral images give information on the color, surface properties, water content and other important physical and chemical properties of the samples (Dissing et al., 2013; Guo et al., 2007). This can be helpful in different fields from food quality control in industry to different biological research areas in academia.

**References for Example 1J**


**EXAMPLE 2. - ANTIFUNGAL METABOLITES FROM LACTIC AND PROPIONIC ACID BACTERIA**

**2.1 Methods for identifying antifungal compounds from ferments**

Work in the area of biopreservation by lactic acid bacteria (LAB) focuses on biological screening for new strains in different applications and discovery work for understanding the biochemistry responsible for the antagonistic effect, especially for identifying antifungal compounds. Numerous compounds from LAB have been assigned as antifungal and accumulated in reviews and textbooks (Crowley, Mahony, and van Sinderen 2013; Schwenninger, Meile, and Lacroix 2011; Dalie, Deschamps, and Richard-Forget 2010). The compounds include inorganics like hydrogen peroxide and carbon dioxide, small (<500 Da) organic compounds as well as proteinaceous material.
The assessment of antifungal ferments typically includes a basic characterization in terms of sensitivity towards pH shift, heat treatment and enzymes like catalase, lipases and proteases (Schwenninger et al. 2008; Valerio et al. 2009; H. Wang et al. 2011).

2.1.1 Bioassay-guided fractionation
For detection of bioactive metabolites, Lavermicocca et al. (2000) employed an elaborate strategy for fractionation, isolation and eventually identification (Lavermicocca et al. 2000). Strom et al (2002) further evolved the toolbox for discovery by applying a strategy of bioassay-guided fractionation (Strom et al. 2002). The strategy typically consists of a solid phase extraction (SPE) pre-concentration of a compound class followed by repeated liquid chromatography based fractionation until one biologically active compound is isolated.

By selecting the sample preparation steps e.g. the phases for SPE pre-concentration and the following liquid chromatography, it is possible to target compounds with specific properties. Reversed phase systems, using a non-polar stationary phase and polar mobile phases, have been employed for several studies scouting for hydrophobic compounds (Strom et al. 2002; Dal Bello et al. 2007; Schwenninger et al. 2008; Ryan et al. 2011; Sjogren et al. 2003; Yang and Chang 2010). In the search for peptides and proteinaceous compounds, a strategy is applied based on fractionation by ultracentrifugation according to molecular weight followed by reversed phase chromatography (Coda et al. 2011; Coda et al. 2008; Rizzello et al. 2011).

Upon isolation, structural elucidation is performed using classical tools like nuclear magnetic resonance and infra-red spectroscopy as well as mass spectrometry.

2.1.2 Comprehensive targeted screening
Comprehensive targeted screening is a more recent strategy in the search for antifungal metabolites. Developments in instrumental capabilities as well as realizing issues with recovery during pre-concentration (Armaforte et al. 2006; Brosnan et al. 2012) form the basis for targeted LC/MS screening of the full ferment with no or limited sample preparation (Ryan et al. 2011; Brosnan et al. 2012; Guo et al. 2012). The combined resolution from the chromatography and the mass spectrometer enables recognition and quantification of a large number of metabolites. The methodology relies on the availability of identified and purified standard materials.

2.1.3 Screening of live microbial colonies
Although not yet applied to LAB and PAB, novel technologies within surface desorption ionization combined with high resolution mass spectrometry demonstrated the ability to monitor the molecular changes on the surface of live colonies. Rather than working in the dilute system of ferments, this technology allows for screening of the dynamics of secreted compounds in response to the local environment (Watrous et al. 2012; Hsu et al. 2013).

2.2 Compounds from LAB and PAB identified as antifungal

After isolation, identification and/or recognition, the inhibitory potential of identified compounds is evaluated by determination of their ability to inhibit one or more species of interest at a given concentration, the minimal inhibitory concentration (MIC). The MIC is defined as the lowest concentration that will inhibit the visible growth of a microorganism after overnight incubation (Andrews 2001). Table 2.1 lists a range of metabolites from LAB, PAB and co-cultures where both MIC and produced levels in ferments have been reported.

Table 2.1 Selected metabolites found in antifungal bacterial cultures based on LAB, PAB and LAB+PAB with concentrations observed in ferments and reported minimal inhibitory concentrations (MIC)

<table>
<thead>
<tr>
<th>Antimicrobial compound</th>
<th>Observed level [g/L]</th>
<th>MIC [g/L]</th>
<th>Indicator organism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diacetetyl</td>
<td>0.049</td>
<td>-</td>
<td>-</td>
<td>(Suomalainen and Mäyrä-Mäkinen 1999)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt;0.100</td>
<td>Yeasts and molds</td>
<td>(Jay 1982)</td>
</tr>
<tr>
<td>Formic acid</td>
<td>0.065</td>
<td>0.9</td>
<td>Fusarium sp.</td>
<td>(Corsetti et al. 1998)</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>0.8</td>
<td>0.5</td>
<td>Fusarium sp.</td>
<td>(Corsetti et al. 1998)</td>
</tr>
<tr>
<td></td>
<td>10⁶</td>
<td>3.0 – 30</td>
<td>Candida sp.</td>
<td>(Schwenninger et al. 2008)</td>
</tr>
<tr>
<td></td>
<td>2.1⁸</td>
<td>1.8 – 30</td>
<td>Yeasts and molds</td>
<td>(Lind et al. 2005)</td>
</tr>
<tr>
<td>Propanoic acid</td>
<td>0.01</td>
<td>0.6</td>
<td>Fusarium sp.</td>
<td>(Corsetti et al. 1998)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt;0.4</td>
<td>Candida sp.</td>
<td>(Doores 2005)</td>
</tr>
<tr>
<td></td>
<td>7.8⁸</td>
<td>1.5 – 45</td>
<td>Yeasts and molds</td>
<td>(Lind et al. 2005)</td>
</tr>
<tr>
<td></td>
<td>27⁶</td>
<td>-</td>
<td>-</td>
<td>(Schwenninger et al. 2008)</td>
</tr>
<tr>
<td>Butanoic acid</td>
<td>0.09</td>
<td>0.8</td>
<td>Fusarium sp.</td>
<td>(Corsetti et al. 1998)</td>
</tr>
<tr>
<td>Pentanoic acid</td>
<td>0.01</td>
<td>0.8</td>
<td>Fusarium sp.</td>
<td>(Corsetti et al. 1998)</td>
</tr>
<tr>
<td>Hexanoic acid</td>
<td>0.10</td>
<td>0.5</td>
<td>Fusarium sp.</td>
<td>(Corsetti et al. 1998)</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>n.d.</td>
<td>&gt;45</td>
<td>Candida sp.</td>
<td>(Schwenninger et al. 2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>105</td>
<td>Debarymyces sp.</td>
<td>(Doores 2005)</td>
</tr>
<tr>
<td>Butanedic acid or (Succinic acid)</td>
<td>3.4⁸</td>
<td>24 – 59</td>
<td>Candida sp.</td>
<td>(Schwenninger et al. 2008)</td>
</tr>
<tr>
<td>2-hydroxy-4-methylpentanoic acid (OH-Me-Pe)</td>
<td>-</td>
<td>10</td>
<td>Penicillium sp. and Aspergillus</td>
<td>(Broberg et al. 2007)</td>
</tr>
</tbody>
</table>
a) Amounts produced in PAB ferments; b) Amounts produced in LAB:PA 3:1 co-culture ferments

- not analysed/no data

MIC values vary significantly according to the indicator organism and conditions, e.g. pH and substrate (Davidson and Parish 1989). As an example, the pronounced effect of pH is demonstrated by the shift of MIC of acetic acid versus a range of three yeasts and five molds from >30 g/L at pH 7 over 7.2 g/L at pH 5 to 1.8 g/L at pH 3 (Lind, Jonsson, and Schnurer 2005).

The dramatic change in MIC with pH suggests the mode of action could be related to the undissociated form of the acid, and with the potency of small organic, fairly apolar acids to penetrate the membrane (Schwenninger, Meile, and Lacroix 2011) Fermentations with lactic acid and propionic acid bacteria typically do not reach pH lower than 3.5 and 4.4, respectively (Lind, Jonsson, and Schnurer 2005).
Besides the range of metabolites listed in table 2.1, a large number of proteinaceous compounds with antifungal properties have been isolated as compiled in recent reviews (Crowley, Mahony, and van Sinderen 2013; Schwenninger, Meile, and Lacroix 2011). Far less information on produced levels and MIC values exists at present.

Inspection of table 2.1 highlights the discrepancy between MIC and produced amounts. Except acetic, propanoic and coriolic acid, the compounds listed are all produced at levels far below the MICs. Even though the compounds observed are antifungal they cannot by themselves be responsible for the overall inhibitory effect. Remembering that an inhibition is actually observed when exposing fungi to ferments, potential explanations for the effect can be deduced:

- One or more potent bioactive compounds are still to be discovered.
- The effect could be synergistic, caused by the combined presence of several of the known, and perhaps still unknown metabolites as suggested by several groups (Corsetti et al. 1998; Lavermicocca et al. 2000; Zhang et al. 2010; Schwenninger et al. 2008; Ryan et al. 2011; Guo et al. 2012).
- Depletion of essential nutrients for the fungal growth could together with produced metabolites provide the effect.

Further work on bioassay-guided fractionation could provide new candidates. In the case of a combined effect, the bioassay-guided fractionation is less suited, as the stronghold of this method is the isolation and identification of single active components. Consequently, it is likely that combined or correlated effects will be lost or overlooked in the process.

All in all, LAB, PAB and LAB + PAB cultures have been shown to have antifungal effect in a wide range of applications. A number of antifungal metabolites have been identified in the ferments, but the causal link between the ferment composition and the antifungal effect remains to be established.

EXAMPLE 3 - MICROBIAL METABOLOMICS

3.1 Terms and definitions
Metabolites are molecules involved in metabolism with a molecular weight less than 1500 Da. The exact definition of the upper Mw limit is less important than to clearly point out that classes of cell macromolecules like genomic material (DNA, RNA), proteins, and polysaccharides are not considered metabolites. Instead, metabolites include a wide variety of organic and in-organics, like
amino acids, carbohydrates, vitamins, minerals and organic acids. They serve as building blocks for cell macromolecules, as the intermediates in cellular process and for providing the energy needed in metabolism. Finally, the metabolites also compromise reaction endpoints and secreted secondary metabolites (Villas-Boas 2007).

The term 'metabolomics' was introduced by Fiehn in 2001 as the comprehensive study of all the compounds involved in cellular metabolism (O Fiehn 2001). Hence, the metabolome is defined as the complete set of all metabolites. In the post-genomics era, the approach supplemented the existing Omics' techniques, e.g. proteomics and genomics for understanding cellular mechanisms. In other words, the genome describes what could happen; the proteome how this would happen and the metabolome what did actually happen.

Metabolomics can be conceived as the extension of the Omics' hierarchy of central dogma of molecular biology with the metabolome as the sum of cellular functions and, importantly, the effect of and on the environment (figure 3.1)

The metabolome represents a large diversity in compound classes and hence chemical properties. Adding to the complexity, some metabolites will be present in mM concentrations whereas some will be present only as a few molecules per cell (Roessner 2007). At any given time point, the metabolome represents a snapshot of all cellular processes. Even when narrowing the scope to a microbial cell, the metabolome consists of several hundreds of compounds impossible to analyze with one method (van der Werf et al. 2007). Accordingly, the metabolome is subdivided into the intracellular metabolites (the endometabolome) and the extracellular metabolites (the exometabolome). These definitions as listed in table 3.1 are useful for microbial systems where cells can be separated from the extracellular environment, e.g. the growth medium.

Table 3.1 Terms and definitions used herein. Adapted from (Mapelli, Olsson, and Nielsen 2008; Nielsen 2007; Oldiges et al. 2007)

| Metabolites | Molecules, typically less than ca. 1500 Da used by or synthesized by a cell during its metabolism |
| Endometabolome | Intracellular metabolites |
| Exometabolome | Extracellular metabolites, i.e. metabolites excreted from the cells and the extracellular environment, typically fermentation media |
| Metabolome | The complete set of metabolites compromising both the endometabolome and the exometabolome |
| Metabolomics | Workflow to comprehensively analyze the full or a specified fraction of the metabolome |
Metabolic profiling | Quantitative or semi-quantitative analysis of a group of metabolites e.g. belonging to a class of compounds or a specific pathway
---|---
Metabolic footprinting | Semi-quantitative or quantitative analysis of the exometabolome, i.e. exometabolites and the components of the growth medium, typically performed as an untargeted study
Targeted analysis | Quantitative analysis of a number of designated compounds
Untargeted analysis | Comprehensive semi-quantitative analysis of all compounds detectable with the selected method of analysis

The scope of a metabolomic study has a severe impact on the strategy selected, especially in terms of sampling and analytical approach. Studies including the full metabolome or only the endometabolome require meticulous experimental planning. Intracellular metabolism is characterized by turnover rates of metabolites in the range of mM/s, i.e. a half-life in the order of seconds or less. To be able to observe relevant changes, all metabolic processes have to be stopped or quenched in the same timeframe. The combined sampling and quenching has to be completed in less than a second. While metabolism remains arrested, the endometabolome is extracted for analysis (Allwood et al. 2013; Villas-Boas 2007; Mashego et al. 2007; van Gulik 2010).

For most extracellular studies, the main source of variability is the presence of living cells as they are responsible for nutrient consumption, secretion of extracellular enzymes and secretion of reaction end products. In the exometabolome, compounds are highly diluted compared to the endometabolome, and turnover rates are reduced accordingly. With the absence of cells, the exometabolome changes only due to extracellular enzyme activity, chemical degradation and reactions (Villas-Boas 2007).

Ideally, metabolomics would be the identification and quantification of all metabolites. As mentioned, this requires a substantial effort by a range of analytical methods. Furthermore, identification (or recognition) and absolute quantification relies on standard materials. Often a subpart of the metabolome, e.g. a specific pathway is observed. Such a targeted approach, which requires a limited number of standards and analytical methods, is designated metabolic profiling.

These types of studies convert into physiological information and the data incorporates into metabolic models (Lahtvee et al. 2011; Adamberg, Seiman, and Vilu 2012). A distinct restriction of this approach lies in only observing the already known metabolites, hereby at the risk of overlooking relevant new compounds (Cameiro et al. 2011).
Untargeted studies rely on comprehensive screening using one or several instrumental platforms. Although untargeted, it is critical to consider that selected analytical methods will detect relevant metabolites and in relevant concentrations. The approach is data driven and is applied when a biological effect is observed but may remain unexplained by analysis of data acquired in targeted analysis. The scope of untargeted studies is often to classify organisms or conditions (e.g. phenotyping), to identify potentially new metabolites and/or build new hypotheses for a biological system. The analysis is normally semi-quantitative and only a selected number of compounds assigned with importance may be identified. A distinction is made between the untargeted analysis of the exometabolome and the endometabolome, which are named metabolic footprinting (Allen et al. 2003) and fingerprinting, respectively (Nielsen 2007; Mapelli, Olsson, and Nielsen 2008).

The potential of exometabolomic footprinting and profiling, especially in relation to investigation of microbial interspecies interaction, is discussed in section 3.4. Focus in this description is on metabolic footprinting of bacterial (i.e. profiling of the exometabolome) fermentations. Experiences and considerations relevant to this description are given in Examples 4, 5 and 6.

3.2 Instrumental platforms

Developments in performance of high resolution analytical instrumentation created the technical foundation for metabolomics. Metabolomics studies rely on sensitive, high resolution instrumentation which has the ability to simultaneously (semi-) quantify and recognize a large number of compounds. Essential instrumental principles of detection are mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR).

Mass spectrometers can be employed as is, e.g. by direct infusion or hyphenated with a frontend based on separation techniques like capillary electrophoresis (CE), gas chromatography (GC) or liquid chromatography (LC) to further enhance the number of detected compounds (G. Theodoridis 2011; G. A. Theodoridis et al. 2012).

The instrumental platforms can be compared by several parameters like sensitivity, number of compounds detected and throughput. As illustrated in figure 3.2, LC/MS is perceived as the best performing analytical technology providing the most optimal combination of potentially thousands of metabolites with the sensitivity down to low nM. Measured on these parameters, NMR appears as the poorest performing method with a lower limit of detection at ca. 1-5 μM. However, NMR offers quantitative sample analysis without any prior separation or derivatisation and with the abilities to identify new metabolites (Wishart 2008; Wishart 2013). As an example of the potential
for NMR in microbial metabolic footprinting, the differences in time course nutrient uptake by four bacterial strains were demonstrated (Behrends et al. 2009).

The difference in the capabilities of GC/MS and LC/MS depicted in fig 3.2 should be conceived as a generalization when considering multi-component methods, especially for untargeted studies. The superiority of either of the techniques in terms of sensitivity and coverage has been demonstrated in various applications. The two most important distinctions between the techniques are the separation mechanism of their frontend and their mode of ionization prior to mass spectrometry. GC is the separation of volatiles, where the separation window is expanded by employing a thermal gradient. Unless the metabolite is volatile by nature, derivatisation is needed prior to analysis. The disadvantages for GC are the risk of thermal degradation and the inherent need for derivatisation for most metabolites (Koek et al. 2011).

On the other hand, GC/MS contains excellent chromatographic resolution in combination with an electron impact being a highly efficient ionization resulting in good quantification performance (Wishart 2013; Oliver Fiehn 2008). Being a robust and mature technology, GC/MS is an indispensable complement to LC/MS and is included in published protocols for microbial metabolomic profiling (Smart et al. 2010; Liebeke et al. 2012).

LC/MS may be considered the broadest applicable technique as there is no need for pre-analysis derivatisation and less risk of degradation of thermally unstable compounds. Although a wide range of compounds can be covered by one LC mode, sets of complementary LC modes are often used (Bajad et al. 2006; van der Werf et al. 2007; Bijscher et al. 2009).

Compared to GC/MS, limitations for LC/MS reside in the aspects of ionization. There is a major inter-compound variation in ionization efficiencies between the different commonly applied atmospheric pressure interfaces, like electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) as well as in between the polarities applied. ESI is the most commonly applied interface, and data acquisition is performed both in positive and negative mode to enhance compound coverage. More details on selection of instrumentation, chromatographic modes, interfaces and their polarities for LC/MS based metabolic footprinting can be found in Honore et al (2013, Anal Bioanal Chem. 2013 Oct;405(25):8151-70).

### 3.3 Workflow of untargeted metabolomics
The overall workflow for an untargeted metabolomics study is outlined in figure 3.3 as described in several reviews (Hendriks et al. 2011; Boccard, Veuthey, and Rudaz 2010; Patti 2011). The overall scope of the process is to answer or build a hypothesis for the biological question raised. The approach is untargeted or data driven, i.e. not based on the measurement of specific pre-selected metabolites.

The study design should span relevant biological variations due to an intervention (Sue et al. 2011), time series (Carneiro et al. 201; Behrends et al. 2009), substrate variations (Carneiro et al. 2011) or comparative studies of strains or phenotypes (Allen et al. 2003). Variations in the metabolite profiles will be caused by the intended biological variation as well as noise and bias. Sampling timing, frequency and numbers of replicates should be selected according to the scope of the study. A priori knowledge of the system of interest, e.g. a fermentation profile can support the design of the sampling protocol. In spite of this knowledge, it is challenging to determine an appropriate number of biological and technical replicates. The system response (here, inhibition of mold) is expected to be the result of multiple correlated changes in metabolites, and hence standard theory for a priori optimal experimental design based on univariate responses becomes less applicable (Hendriks et al. 2011).

Data acquisition in microbial metabolomics typically includes the process from sample generation over sampling, sample handling and collection of analytical data and metadata descriptive of the study, e.g. pH, dry matter. A vital part of the data acquisition is to include a quality control strategy (Dunn et al. 2011; Zelena et al. 2009; E. J. Want et al. 2010; Sangster et al. 2006; Koek et al. 2011), as non-targeted studies do not allow for classical analytical method quality assurance measures as e.g. described by Funk and coworkers (Funk, Damman, and Donnevert 1995). The analytical methods should cover the range of compound classes anticipated to be responsible for or linked to the biological variation. Based on prior knowledge of known metabolites, methods can be selected and tested for covering the compound classes and relevant concentrations. Alternatively, methods can be assessed on their ability to detect biologically observed differences in existing sample sets. For each sample, the data acquisition step results in a large number of data points positioned typically in a two (retention time and mass to charge) dimensional matrix. Experiences and considerations in terms of data acquisition including quality control measures for this description are described in Example 5.

The data processing step includes three major subparts, pre-processing, mining and identification of biomarkers. Pre-processing reduces the dimensionality of the sample data matrices. Due to the
combination of expected complexity of metabolomics samples and limitations in chromatographic resolution, chromatography/mass spectrometry data will contain co-eluted compounds. By the aid of software packages like XCMS (Smith et al. 2006) or MZmine (Pluskal et al. 2010), pre-processing and deconvolution of the three-dimensional data result in an extraction of mass-to-charge \(m/z\) and retention time (rt) information into compound identifiers (features). During this process the data is filtered to exclude noise and features are aligned based on \(m/z\) and rt criteria.

Data mining, often as multivariate data analysis (MVA), is applied to identify treatment differences or discover patterns in the preprocessed data. Non-supervised exploratory methods like principal components analysis (PCA) serve to represent multivariate data into a lower dimensional space. The multivariate data analysis also provides tools for selecting the fraction of the features that have the highest correlation with relevant patterns observed for the samples. These features can then potentially be designated biomarkers.

The next step in the workflow is to assign a chemical identity to the biomarkers. With data acquired on high resolution LC/MS instruments, the observed charge state, adducts, accurate mass and relative isotope ratios can be converted into educated guesses of metabolites estimated on elementary composition (Kind and Fiehn 2010; Kind and Fiehn 2007). MS/MS or MS° data and use of databases of metabolites (Go 2010) can further support the recognition of a chemical compound, which can then be verified with an authentic standard material. Real de novo identification requires isolation of the compound and the combined efforts of both spectrometric and spectroscopic techniques.

The remaining challenge lies in the biological interpretation by correlating the data into metabolic pathway maps, e.g. Kyoto Encyclopedia of Genes and Genomes (KEGG) or BioCyc (Kanehisa and Goto 2000; Caspi et al. 2010). Targeted analysis for determination of concentrations of biomarkers could lead to testing whether the observed correlations with biological activity can be translated into causal information or a hypothesis for new experiments.

3.4 Potential(s) embedded in microbial exometabolomics to study antifungal mechanisms
The mold growth inhibition generated by bacteria is the result of microbial interspecies interactions. A range of microbial interactions has been studied and proposed (figure 3.4). Some of these rely on cellular contact, but the major part takes place without physical contact. Contact- independent metabolic exchange occurs through metabolic exchange factors, which include exometabolites, secretion of genetic or proteinaceous materials and even exchange of virus in the form of
bacteriophages. The production, secretion and presence of exchange factors regulate the metabolism of the individual community member and hereby the overall ecology of the microbiological system. Although sensing mechanisms are recognized as very important, nutrient availability is mostly responsible for shaping the microbial community (Phelan et al. 2012).

Recent exometabolomic studies have provided new insights on the effects of nutrient availability in microbial mono-cultures. During the growth phases of batch fermentation, extended overflow mechanisms regulate secretion and uptake of normally intracellular metabolites. In the Carbon-source nutrient excess situation, selected normal intracellular metabolites are secreted to be re-uptaken after depletion of the Carbon-source (Paczia et al. 2012). The findings indicate a competitive strategy for one microorganism to outcompete other strains in nutrient-rich environments. Moreover, the observations will have impact on the definitions of the endo- and exometabolome as well as for coming metabolomics experiments. Similar studies of *Escherichia coli* strains likewise illustrate secretion of metabolites that participate in a wide range of pathways as a response to environmental conditions. Additionally, the exometabolome proved more complex than expected, which supports the need for un-biased non-targeted studies (Carneiro et al. 2011).

Exometabolomic studies allow for investigation of the mode of action of antimicrobial substances. By applying sublethal doses of fungicides to yeast, direct infusion MS metabolic footprinting classifies the fungicides according to their effect on the metabolism, and hence mode of action (Allen et al. 2004).

In a recent similar work based on NMR, the mode of action of the antimicrobial triphenyl-bismuthdichloride against *Staphylococcus aureus* was investigated. By combination of exometabolomic and enzymologic studies, specifically the antimicrobial effect was due to the inhibition of bacterial pyruvate dehydrogenase complex (Birkenstock et al. 2012).

The sensitivity of GC/MS exometabolomic studies was demonstrated by detecting microbial contamination of microalgae fermentation only three hours after intended contamination and assigning the compounds responsible for the detection (Sue et al. 2011).

Metabolic footprinting could serve as a powerful supplement for investigation of the antifungal properties of bacterial ferments. The global analytical approach includes the full exometabolome, hereby considering both formed and depleted metabolites. Working with an untargeted approach, potentiates the discovery of previously non-discovered metabolites. The challenge lies within
systematic variation in the bacterial fermentations to induce changes in mold growth. The data mining workflow would serve for finding correlations between the observed bacterial profiles and mold growth, which again could lead to identification of antifungal compounds.

To cite Kell et al, 2005 (Kell et al. 2005):
"Metabolic Footprinting and Systems biology: The medium is the message"

EXAMPLE 4 - BIOLOGICAL STUDY DESIGN

4.1 Experimental strategy
The central dogma of the work was that changes induced in the exometabolome of the LAB would lead to differences in mold growing on the spent media. Comparative untargeted analysis of the metabolome should lead to the discovery of discriminating, bioactive compounds. To be able to correlate the observed mold growth with the chemical analysis, the sample preparation of the ferments should be kept at a minimum and be similar both for the chemical analysis and mold growth assay as outlined in fig 4.1.

4.2 Deconvolution of the system
It requires a complex system to observe the inhibition of fungi by a culture with antifungal properties in yoghurt.

From a microbiological viewpoint, the system is made up by the starter culture (typically consisting of two or more LABs), the culture with antifungal properties (again in this case, two bacterial strains) and the contaminant (e.g. mold). Each class of organisms has different properties e.g. in terms of growth rate. To add to the complexity, different strains of molds and yeasts respond differently to the cultures with antifungal properties.

Prior to inoculation with the two cultures, the main component of the system is bovine milk, a water-based fluid, composed of proteins, lipids, carbohydrates, vitamins and minerals. Each of the compound classes represents a wide diversity of compounds. Furthermore, the constituents are present either in aqueous solution, as colloidal solution (single molecules or aggregates) or as an emulsion. During the growth of the lactic acid bacteria, lactose is fermented to lactic acid, which causes an acidification to below pH ca. 4.6. The shift in pH below this point results in a change of the colloidal stability, and casein micelles aggregate or gel (Fox 2009). Furthermore, extracellular
proteases from LABs expand the chemical complexity by enzymatic hydrolysis of proteins. Overall, the growth medium shifts from complex to more complex during the fermentation.

The number of potential variations in the system needed to be reduced to be able to monitor and relate chemical changes in the exometabolome to biological observations. Hence, the strategy was to apply the exploratory data-driven approach in a model system and then validate findings in the food system by targeted analysis. The simplification was obtained by exchanging the milk/yoghurt with a chemically defined media. Additionally, the number of microbial species were reduced by excluding the yoghurt starter organisms and limiting the number of indicator organisms and only apply these for in vitro tests (table 4.1).

**Table 4.1 Comparison of components in food and model systems**

<table>
<thead>
<tr>
<th>System component</th>
<th>Confirmatory food system</th>
<th>Exploratory model system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate</td>
<td>Milk/yoghurt, i.e. non-defined in term of protein and lipids Heterogenous, phase shift</td>
<td>Chemically defined media, a transparent one phase system</td>
</tr>
<tr>
<td>Starter culture</td>
<td><em>Streptococcus thermophilus</em> <em>Lactococcus delbrückii</em> subsp. <em>bulgaris</em></td>
<td></td>
</tr>
<tr>
<td>Culture with antifungal properties</td>
<td><em>Lactobacillus paracasei</em> <em>Propionibacterium freudenreichii</em> subsp. <em>shermanii</em></td>
<td><em>Lactobacillus paracasei</em> <em>Propionibacterium freudenreichii</em> subsp. <em>shermanii</em></td>
</tr>
<tr>
<td>Target organisms (contaminants)</td>
<td>Multiple yeasts and molds with varying sensitivity</td>
<td>Two mold strains</td>
</tr>
<tr>
<td>Test systems</td>
<td><em>in vitro</em>, spot tests</td>
<td><em>in vitro</em>, spot tests</td>
</tr>
<tr>
<td></td>
<td><em>in vivo</em> tests, e.g. long term storage</td>
<td></td>
</tr>
</tbody>
</table>

A range of *Lactobacillus paracasei* strains with different antifungal properties were included to examine the variation of their metabolism and the resulting effect on mold growth. Details on the organisms applied during the project and their abbreviations are listed in table 4.2.

**Table 4.2 List of microorganisms and the applied abbreviations as used herein**

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Group</th>
<th>Strain</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria</td>
<td>Lactic acid bacteria (LAB)</td>
<td><em>Lactobacillus paracasei</em></td>
<td>LAB A</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Lactobacillus paracasei</em></td>
<td>LAB B</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Lactobacillus paracasei</em></td>
<td>LAB C</td>
</tr>
<tr>
<td></td>
<td>Propionic acid bacteria (PAB)</td>
<td><em>Propionibacterium freudenreichii</em> subsp. <em>shermanii</em></td>
<td>PAB A</td>
</tr>
<tr>
<td>Fungi</td>
<td>Mold</td>
<td><em>Penicillium solitum</em> DCS 302</td>
<td>DCS 302</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Penicillium</em> sp. DCS 1541</td>
<td>DCS 1541</td>
</tr>
</tbody>
</table>

4.3 Defining and building the toolbox
In order to employ the decided strategy to investigate the antifungal mechanisms, the relevant tools had to be developed and validated.

Firstly, a suitable chemically defined media should be constructed, tested for growth of all involved species and for inhibition. Besides the biological aspects, the media should be suited for chemical analysis and not contain non-chemically defined components, like yeast extract or surfactants (e.g. TWEEN 80). The development and validation of the chemically defined interaction medium (CDIM) is described in Example 1F.

The growth of the mold served as the biological response, i.e. the dependent variable. The morphological changes during mold growth are not only described by colony size (area) but also by e.g. color. The variable should constitute objective, preferably metric and ratio scale data. The documentation of mold growth was made by multispectral imaging as described in Examples 1G and 1H and growth characteristics data was extracted by a clustering algorithm described in Example 1J.

The relative changes in the ferments should be mapped comprehensively and unbiased. The instrumentation and methods for the LC/MS analysis were selected based on expected bacterial metabolites (Honore et al. 2013 Anal Bioanal Chem. 2013 Oct;405(25):8151-70). GC/MS methods were selected to provide the analysis of small organic acids and volatiles, which were not covered by the LC/MS methods. The methods were applied in Examples 1G and 1H.

4.4 Combining the tools - Experimental set-up for metabolomics experiments
The project work is based on the iterative process of exploring microbiology and analytical chemistry. Hence, observations and conclusions are drawn from both disciplines. Prior to performing each actual metabolomic experiment, a range of microbiological screening experiments was conducted to define experimental conditions like inoculation amounts, fermentation time, and where relevant, mold growth as response to the conditions.

With the defined biological conditions, experiments were constructed to generate samples and collect a full dataset, typically as outlined in figure 4.2.

The experiments started with batch fermentation of LAB with variations in substrate or/and strains. The parallel fermentations were monitored for pH, volatiles in the headspace development of the culture (measures like colony forming units, optical density and biomass).
During and after the end of fermentation, samples were withdrawn, cooled and divided into two subsets, one with and the second without LAB cells. Mold growth assay was performed on both sets. During the incubation period, mold growth was monitored by multispectral imaging and presence of volatiles was measured by headspace GC/MS. From the subset of cell-free ferments (exometabolome) a set of multiple aliquot was frozen and stored for subsequent analysis.

Details and considerations in terms of each step of the data acquisition (i.e. from sample LAB fermentation to acquiring chemical raw data) are given in Example 5.

**EXAMPLE 5 - DATA ACQUISITION**

**5.1 Sample generation - LAB and PAB fermentations**

The purpose of the bacterial fermentations was to generate ferments with relevant variations in relation to biological properties, i.e. antifungal effect. The selection of conditions and execution of experiments proved critical in order to reduce non-relevant variations, i.e. biological ‘noise’.

When working with LAB, two aspects proved to be critical, namely history of the inoculants and cell concentration. Cultures should be pre-fermented in and harvested from the same liquid medium prior to batch fermentation otherwise differences in lag time were readily observed. Measuring and controlling cell concentrations of inocula proved challenging, especially in between *Lb. paracasei* strains, as they demonstrated different degrees of agglomeration as observed by microscopy. As a consequence, optical density (OD) was adopted for standardizing inoculants and biomass for supplementing plate counts (colony forming units).

Bacteria were batch-fermented in CDIM in flasks, i.e. sterile medium was inoculated with bacteria and fermentation progressed at a given temperature to the endpoint, either based on pH or time.

Batch fermentation is characterized by a given pool of nutrients at start, accumulation of metabolites and biomass. This corresponded to the original application (fermented milk), where the starter culture and the culture with antifungal properties ferment the milk to pH 4.6 at 42°C. The fermented milk is then cooled to 5°C.

An alternative to batch fermentation could be fermentation in a bioreactor. Fermentations in a bioreactor or fermentor can be controlled in terms of a range of key parameters like pH (dosage of
acid/base), nutrients, atmosphere, stirring and biomass. Bioreactors are typically fitted with a range of sensors (e.g. pH, dissolved oxygen, temperature), which both serve for control but also for monitoring. Finally, bioreactors come with sampling ports that enable repeated, fast and sterile sampling (van Gulik 2010). Control of the key parameters makes bioreactor fermentations more reproducible than batch fermentations. Hence, fermentations in bioreactors are ideally suited for flux experiments, time-course and endometabolomic studies (Allwood et al. 2013). Due to the cost of investment and complexity in use, experiments with bioreactors are limited in terms of number of replicates. As untargeted studies are based on comparisons, mainly of endpoint of fermentations, batch fermentation was considered the optimal choice. Using batch fermentations allowed for including multiple parallel combinations of conditions and biological replicates in each experiment (Examples 1G and 1H). The limitation in the number of combinations was in the manual sample handling and number of available electrodes for continuous monitoring and acquisition of pH in the used CINAC equipment (fig. 5.1).

An example of batch fermentation profiles is shown in figure 5.1. For LABs, pH is an excellent measure of fermentation progress, as it is correlated to both nutrient consumption and formation of biomass. As an example, LAB C displayed the phases of batch fermentation with an initial lag phase (ca. 0 - 10 hours), followed by an exponential growth phase (ca. 10 - 30 hours) followed by a stationary phase.

The production of volatiles was estimated during fermentation in the headspace of small aliquots (2 ml) of inoculated CDIM in 20 ml headspace vials in the thermostated (same temperature as fermentations) autosampler of a GCMS instrument (Examples 1G and 1H). Discussion of the headspace methods is found in section 5.5.

5.2 Sampling and storage
A key aspect of the experimental strategy was to be able to correlate the metabolic footprint with the biological response. The biological response was obtained by spotting indicator molds onto LAB ferments with (C) or without bacterial cells (CF) solidified with agar. No actions (heating, precipitation, addition of proteases, molecular weight filtrations) were taken to inactivate any potential residual enzymes. The test for mold growth was typically observed for up to nine days at 25°C. During this period, composition of both C and CF changed based on presence of bacterial cells and enzymatic/chemical reactions. Studies in richer medium, milk, showed that added bacteria remain viable up to nine days (Kruse Jensen, non-published results).
In spite of this challenge of a long term test, efforts were made to minimize the biological variance during sampling.

To minimize the biological variation during sampling, fast quenching could be ideal. Quenching is the process of stopping metabolism by dramatically altering the temperature and/or pH by instantaneous mixing of the sample with a pre-heated (>80°C)/pre-cooled (< -40°C) quenching solution, e.g. 60/40 methanol/water v/v. The technique allows for complete sampling and quenching within less than a second, which is a prerequisite for studies of the endometabolome, where, as mentioned earlier, metabolite turnover rates are in the regime of a few mM/s (Villas-Boas 2007).

For the current study, a quenching protocol conflicted with the scope of the study due to:
- Risk of leakage of metabolites
- Need for removing quenching solution prior to the mold growth assay

Quenching of the cells results in leakage of intracellular metabolites (Spura et al. 2009; Schadel, David, and Franco-Lara 2011; Wellerdiek et al. 2009; Bolten et al. 2007). The degree of leakage depends on the ruggedness of the type of cell towards the quenching protocol, with bacterial cells, and especially gram-negative being the most sensitive (Villas-Boas 2007). The leakage occurs both due to the dramatic change in osmotic environment and temperature which negatively influences the cell membrane integrity (Schadel, David, and Franco-Lara 2011). Due to the risk of leakage, recent exometabolomic protocols rely on fast and cold filtration or centrifugation (<5°C) for removal of cells prior to quenching e.g.(Liebeke et al. 2012; Paczia et al. 2012).

As changes in the exometabolome occur on a much slower scale, the procedure for separating cells from the medium at sub-ambient temperature was selected. At sampling points, aliquots of maximum 50 ml were rapidly transferred to ice-water at 0°C. Samples intended for CF studies were then centrifuged at 0°C and filtered. After cell removal, multiple aliquots were frozen on dry ice and stored at -80°C until different types of profiling and targeted analyses. Sampling of multiple aliquots allowed for different types of analyses on the same ferment without the need for repeated thawing and freezing. Sample aliquots was considered stable for up to 6 months when stored at -80°C (Gika, Theodoridis, and Wilson 2008b). Bacterial ferments (C and CF) were kept at 0°C until heated to 48°C for preparing agar plates for mold growth tests.

5.3 Characterization of mold growth
The growth of mold served as the primary biological response variable (Examples 1F, 1G and 1H). The growth assay was performed as an in-vitro spot test on ferments with or without LAB and/or PAB cells solidified with agar. Two Penicillium strains were selected as indicator molds as they had been isolated as dairy contaminants and were empirically known to respond differently. Precautions were taken to minimize parameters known to influence the results of methods for testing efficacy of food antimicrobials, like preparation of the test media, the numbers and condition of the test organism, volume of applied test organism suspension, atmosphere and temperature (Davidson and Parish 1989). As an example, non-inoculated CDIM were incubated and handled like fermented samples to serve as references. Rather than affecting the fermented samples with pH adjustment, the references where acidified to evaluate and compensate for the effect of lactic acid and pH shifts. Consequently, the mold growth depended on the consumed amount of nutrients and metabolites formed (including pH shift) by the bacteria.

The original internal method for assessing mold growth is based on measurements of colony diameter. Growth of filamentous fungi like Penicillium has a more complex growth pattern than most bacteria. The growth can be divided into at least five phases starting with lag phase, then first transition period, the log phase a second transition period and ending with the stationary (Meletiadis, Meis, and Mouton 2001). The phases include germination of spores and conidia, elongation of hyphae, formation and maturation of spores.

The pigmentation of Penicillium changes over time from white to green as the colony ages. Besides age, color change can reflect the extent of environmental stress or depletion of a component from the medium (Hanson 2008). This was apparent when observing the growth of a mold colony, which over time and due to modifications in the medium changed in several morphological aspects like size, density and color.

The effect of pH of CDIM titrated with lactic acid on indicator Penicillium solitum DCS 302 was clearly observed based on color, but less apparent measuring the diameter (figure 5.2a). The effect of LAB A cell presence was even more significant, as illustrated in figure 5.2b.

To monitor and document mold growth under controlled conditions, multispectral images were acquired using a VideometerLAB 2 (Videometer A/S, Hørsholm, Denmark). Images were collected from start of visual growth (day 2) and continued on a daily basis as described in Examples 1G and 1H. The multispectral dataset allows for analyzing the mold colonies in the spectrum wavelength range from ultraviolet to near-infrared (375 - 970 nm). The data could be processed into visual
images similar to typical Red-Green-Blue (RGB) photos obtained by commercial digital cameras (fig 5.2).

Furthermore, the data could be processed for changes occurring outside the visual spectrum (ca. 390-700 nm).

The data acquisition method was intended for both mold colonies on the transparent CDIM, CDIM with bacterial cells and for application studies on fermented milk. The backlight channel data from the instrument is extremely useful for determination of colony sizes, but is not suited for non-transparent samples like fermented milk.

Instead, Ebrahimi et al. (Example 1J) developed an algorithm for differentiating mold colonies from the media, extracting spectral information and clustering the colony into two color classes, white and green. The number of clustered pixels then represented the mold growth when subjected to different conditions. Depending on the effect of the medium, the variations in mold growth were assessed by including one or more of the clusters. The minor variation spanned due to different pH (fig. 5.2a) on different days was best described by green pixels as shown in figure 5.3a. In contrast, the more pronounced variation observed with and without presence of LAB A cells over time was best described using the sum of white and green pixels (figure 5.3 b).

Volatilees proved to be of relevance to mold growth in fermented CDIM with (C) and without (CF) LAB cells. Two parallel sets of agar plates were prepared and handled similarly. One set was subjected to mold spot test and the other was used for sampling of agar plugs. Plugs were frozen at -18°C and collected until the end of an experiment. The agar plugs were analyzed by headspace sampling gas chromatography as described in Example 1G and in section 5.5.

5.4 Metabolic footprinting of LAB and PAB ferments by LC/MS

Untargeted studies of the full microbial metabolome require the combined efforts of several analytical platforms to cover the variety of compounds present (van der Werf et al. 2007). When focusing on the exometabolome, a number of highly polar/charged intracellular metabolites are excluded, which narrows the analytical scope. Based on the limitations expected from using a combination of reversed phase (RPC) and hydrophilic interaction liquid chromatography (HILIC) with MS detection (Honore et al 2013 Anal Bioanal Chem. 2013 Oct;405(25):8151-70). both LC/MS and GC/MS instrumental platforms were included. However, new methods were included during the project according to the needs and problems that appeared.
5.4.1 Sample preparation
As the chromatographic profile should closely resemble the ferment used in the mold growth assay, sample preparations was kept at a minimum. Additionally, studies with pre-concentration of ferments using reversed phase solid phase extraction show poor recoveries of known metabolites and hereby recommend analysis of the full ferments (Armaforte et al. 2006; Brosnan et al. 2012). Samples were diluted (5x) in a suitable solvent containing stable isotope labeled amino acids phenylalanine and leucine. The two amino acids were well-retained both in RPC and HILIC and detectable in both positive and negative ESI.

Using the 'dilute and shoot' protocol provided a holistic profiling, but only of the most abundant/best responding components. The sensitivity of the method was limited by how much sample could be loaded onto the column. This was again restricted by the composition of the matrix, especially by inorganic salts and both well-responding and abundant amino acids.

5.4.2 Selection of chromatographic conditions
Selection of chromatographic modes and instrumental platform with regard to LC/MS was made according to considerations of the expected exometabolome (Honore et al 2013 Anal Bioanal Chem. 2013 Oct;405(25):8151-70). The synthetic exometabolome was based on selected nutrients of an initial version of the CDIM and supplemented with selected reported metabolites (table 5.1). However, the synthetic exometabolome did not contain any inorganic salts, like chlorides, sulfates and phosphates.

Table 5.1 Synthetic exometabolome used for initial testing of chromatographic systems

<table>
<thead>
<tr>
<th>Compound class (number included)</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carboxylic acids, short chain (9)</td>
<td>Formic acid(^a), acetic acid(^a), propanoic acid, butanoic acid, lactic acid, succinic acid, 2-hydroxy-3-phenylpropanoic acid, 2-hydroxy-3-(4-hydroxyphenyl) propanoic acid, Pyrrolidone-5-carboxylic acid</td>
</tr>
<tr>
<td>Biogenic amines (2)</td>
<td>Putrescine, histamine</td>
</tr>
<tr>
<td>Amino Acids (17)</td>
<td>All essential except Cys, Asn, Gln, Met and Trp, but with CysCys added</td>
</tr>
<tr>
<td>Carbohydrate (2)</td>
<td>Lactose, glucose</td>
</tr>
<tr>
<td>Nucleoside and pyrimidine (2)</td>
<td>Uridine, Uracil</td>
</tr>
<tr>
<td>Cyclic dipeptides (2)</td>
<td>Cyclo(Gly-L-Leu), cyclo(Phe-Pro)</td>
</tr>
<tr>
<td>Fatty acids (4)</td>
<td>Octadecenoic acid, dodecanoic acid, 3-hydroxytetramyristic acid(^a), hexadecanoic acid(^a)</td>
</tr>
<tr>
<td>Volatiles (6)</td>
<td>Acetaldehyde(^a), 2,3-butanediol, acetoin(^a), diacetyl(^b), methylglyoxal(^a), glyoxal(^a)</td>
</tr>
</tbody>
</table>
Oligopeptides (1) Nisin A
a) Not included in testing of LC/MS

Small polar organic acids (like formic, acetic and propanoic acid) and carbonyl compounds were not expected to be detected by LC/MS and were addressed by GC/MS. Using the synthetic exometabolome, two HILIC (Amide and ZIC-HILIC) and two RPC (PFP and polar embedded C18) columns were compared for performance in terms of peak shape and run time (table 5.2). The Kinetix PFP was discarded due to significant bleeding (elution of column residues) which could not be reduced even by several rinsing protocols. On the other hand, the polar embedded Waters C18 had an acceptable low background, peak shapes and retention for the lipophilic amino acids and acids, cyclic dipeptides and nisin.

### Table 5.2 Tested columns and conditions

<table>
<thead>
<tr>
<th>Chromatographic mode</th>
<th>Column</th>
<th>Dimensions</th>
<th>Mobile phase additives used for testing</th>
</tr>
</thead>
<tbody>
<tr>
<td>HILIC Gradient: From 95/5 to 50/50 v/v acetonitrile/water</td>
<td>Merck Sequant, ZIC-HILIC Amide</td>
<td>150 x 2.1 mm ID; 3.5 µm; 100Å Max pressure 350 bar</td>
<td>10 mM NH$_3$FA, pH 3.5</td>
</tr>
<tr>
<td></td>
<td>Waters Acquity UPLC BEH Amide</td>
<td>150 x 2.1 mm ID, 1.7µm; 130Å</td>
<td>20 mM NH$_3$FA, pH 3.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10 mM NH$_3$FA, pH 5.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.5%v/v FA</td>
</tr>
<tr>
<td>RPC Gradient: From 100/0 to 0/100 v/v water/acetonitrile</td>
<td>Phenomenex Kinetix PFP T3</td>
<td>150 x 2.1 mm ID, 1.7µm; 100Å</td>
<td>0.1%v/v FA</td>
</tr>
<tr>
<td></td>
<td>Waters Acquity UPLC HSS T3</td>
<td>150 x 2.1 mm ID, 1.8µm; 100Å</td>
<td></td>
</tr>
</tbody>
</table>

a) Dimensions stated as length x diameter (mm), particle size (µm) and pore size (Å)
b) NH$_3$FA - ammonium formate, FA formic acid

The two HILIC columns were tested with different mobile phases. Both proved to have acceptable peak shapes for glucose and selected amino acids, but especially peak shape of organic acids was poor in ZIC-HILIC with ammonium formate. Peak shapes of the acids were slightly better in formic acid but at the expense of a poor peak shape for a range of amino acids (data not shown). Both considering the peak shapes and ability to operate in ultra-performance liquid chromatography (UPLC) mode, the most suitable compromise was achieved with the BEH Amide column and ammonium formate.

The optimal solution would have been to optimize chromatographic conditions on actual ferments and use the cumulative scoring system set by Bajad et al, 2006 for a limited number of selected compounds. The optimal choice of conditions could be selected based on the cumulative score of some key chromatographic performance characteristics like sensitivity, peak height, symmetry and
retention (Bajad et al. 2006). However, this requires a-priori prioritization of compounds - and that the CDIM was available at the time of method development.

The performance of the selected chromatographic conditions is shown in figure 5.4 with a QC (mix sample) showing the four chromatographic modes. Injection volumes from 1 to 15 µl were tested and 10 µl was the upper limit in respect to chromatographic performance. The two MS polarities proved to be complementary as described in Example 1H, with positive mode highly dominated by especially amino acids and negative mode by carboxylic acids.

The high concentration of sulfate and phosphate in samples, negatively affected HILIC chromatography quite significantly. Three distinct sets of salt clusters with sulfate and phosphate clusters dominated the HILIC ESI neg in the retention time windows 5-6 min and 6.5-8 min. The robustness of HILIC appeared excellent when using repeated injections of one sample type, e.g. pooled QC samples (MIX or QC samples), even with varying injection volume. During analysis of full sample sets, uncontrolled variations were observed in HILIC. Probably due to insufficient buffering during sample dilution or too weak buffer effect of the mobile phase, the salt cluster profile (sulfate, phosphate) observed in TIC changed dramatically from one injection to the next. The phenomenon was most evident within injection sequences with large shifts in pH, e.g. from an un-inoculated CDIM (pH 6.5) to LAB ferments (pH <4.0).

As the HILIC retention mechanism remains to be fully explained (Buszewski and Noga 2012), no exact explanation for the observation can be given. The shift in degree of protonation of an abundant component like lactic acid could affect the amount of immobilized water rich layer hereby severely shifting retentive properties.

In a study of rat urine, Spagou et al., 2011, demonstrates excellent repeatability of a HILIC UPLC system using a BEH HILIC (i.e. silica) column and mobile phase 0.1% v/v formic acid and 10 mM ammonium acetate in acetonitrile (MeCN) (Spagou et al. 2011). Unfortunately, no details are provided on injection volume and pH of samples and mobile phases. The pronounced effect of pH of the mobile phases on BEH HILIC chromatographic performance is seen in an earlier study, also on rat urine (Gika, Theodoridis, and Wilson 2008a).

Both studies demonstrate that obtaining repeatable HILIC chromatograms in 'dilute and shoot' studies should be feasible upon further optimization, probably with focus on mobile phase modifier pH.
Attempts were made to improve the robustness of the HILIC separation system, e.g. increasing equilibration period and sample dilution and lowering the injection volume, but the problem was not fully resolved.

Due to the instability of HILIC, main focus onwards was on RPC data, as HILIC data in several instances did not pass quality assessments as outlined in the following section.

5.4.3 Quality assurance and data inspection

For targeted analytical work, strategies for ensuring validity of the method are well-described.

This includes the use of appropriate standards, which serve for identifying problems and potential correction for each step of an analytical procedure from storage to detection as excellently described by Koek and coworkers (Koek et al. 201).

In untargeted profiling, these classical strategies for assuring the validity cannot be applied to the same extent. Observations and potential conclusions are typically drawn from comparative studies. It is critical that observed variations arise from relevant variations in the samples and do not originate from errors in study design or are not introduced during sample preparation and instrumental data acquisition, e.g. instrumental drift and/or instability of samples.

This summarizes into two key questions
- How to minimize and monitor the contribution of variation from analytical data acquisition?
- How to evaluate the fitness of a dataset for further analysis?

An important tool for addressing these questions was to include a quality control (QC or MIX) sample, which was a pooled mixture, composed of an aliquot of each test sample in the study (Sangster et al. 2006). The QC sample represented an average sample which served to support data quality prior to, during and after data acquisition. Upon generation, it is important to divide the QC sample in multiple sub-aliquots (e.g. 15 x) to avoid freeze-thaw cycles.

The use of a QC sample supports the quality assurance throughout the data acquisition and provides measures for the optimal post-run processing of data. The potential of and recommendations for using a QC sample has been outlined in papers and protocols (Zelena et al.
A summary of the uses of QC sample data is given in Table 5.3.

<table>
<thead>
<tr>
<th>Timing</th>
<th>Purpose</th>
<th>How</th>
<th>Focus on</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-run</td>
<td>Overall System performance compared to previous study</td>
<td>Include QC sample from previous study in present study</td>
<td>Visual comparison of BPC and TIC for chromatographic resolution and response</td>
</tr>
<tr>
<td></td>
<td>Equilibration of system</td>
<td>Repeated injections prior to analytical sequence</td>
<td>Achieve repeatable BPCs and TICs</td>
</tr>
<tr>
<td>Post-run</td>
<td>Assurance of data quality</td>
<td>Overlay of TICs, BPCs, EICs</td>
<td>Irregularities in retention time, peak shapes and m/z</td>
</tr>
<tr>
<td></td>
<td>Univariate statistics of feature responses (area or intensities)</td>
<td>Distribution of coefficient of variance (CV). Guideline suggests that &gt;70% of the features should have CV &lt; 30% (E. J. Want et al. 2010)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Unsupervised multivariate data analysis (PCA)</td>
<td>a) Tight clustering of QC sample in center of scores plot PC1 and PC2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>b) Inspection for systematic variation of QC in PC1, PC2, PC3 scores vs. run number</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Selection of parameters for feature selection</td>
<td>Select e.g. early, mid and end sequence runs</td>
<td>Tolerances for retention time shift, m/z shift and peak widths</td>
</tr>
<tr>
<td></td>
<td>Correction/normalization</td>
<td>Normalization with internal standards or features from QC sample</td>
<td>Eliminate drift and analytical batch to variations.</td>
</tr>
<tr>
<td></td>
<td>Selection of scaling algorithm</td>
<td>Test algorithms</td>
<td>Reduce heteroscedasticity</td>
</tr>
</tbody>
</table>

Table 5.3 Use of pooled QC samples for quality control and processing of data

When analyzing samples from a study, the injection sequences contained the actual test samples, sample blanks, QC sample from the present study and QC sample from a previous study (QC-old). The injection sequence run order was structured in brackets, each starting with a sample blank followed by a maximum of ten test sample and one QC sample randomized according to study design. Prior to the brackets, QC sample injections (typically ten) and one QC sample injection from previous study were run to ensure proper equilibration of the LC/MS system. The organization of an injection sequence is illustrated in fig. 5.5 Comparison of total ion current (TIC) and base peak (BPC) chromatograms of the QC sample acquired in present and previous study (QC-old) was used for evaluation of LC/MS system performance under the influence of the full matrix.
The use of synthetic QC test samples - or synthetic test mix is a valuable relevant supplement (Zelena et al. 2009; E. J. Want et al. 2010; Koek et al. 2011) but requires thorough construction according to the specific study to visualize relevant overall instrumental performance. When appropriately designed, the test mix serves for identifying performance of the subparts of the LC/MS system (Mutton et al. 2011).

Post-run inspection of acquired TICs, BPCs and selected extracted ion chromatograms (EIC) of the QC sample injections readily revealed any irregularities in retention time, intensities and mass accuracy that would trigger a re-analysis of the sample set. Sample blanks provided information of artifacts from consumables and extent of carry-over.

Passing the basic inspection of raw data files, the next level of data quality control was performed by principal component analysis (PCA) after feature extraction (described in Example 6) without sample blanks and QC-old. The processing and examples of use of QC sample data are discussed in more details in Example 6.

5.5 Gas chromatographic analysis of LAB ferments

Gas chromatography mass spectrometry (GC/MS) is extensively used for metabolomics, both as semi-quantitative profiling and quantitative. Depending of the scope, GC/MS analysis studies are performed without derivatisation (for volatiles) and with derivatisation for less volatile or non-volatile compounds.

5.5.1 Sampling and analysis of the headspace of LAB ferments

Multiple methodologies exist for sampling of volatiles, e.g. by distillation, extraction into a non-water-miscible volatile solvent or by sampling of the headspace over a sample (Parliment 1997; Wampler 1997; Harmon 1997). Sampling and analysis of the headspace of microbial ferments provide an opportunity to investigate volatile metabolites almost independent of the sample matrix and with a minimum of preparation.

The headspace can be principally sampled in three different ways, depending on the restrictions and requirements in terms of sampling time, sampling amounts and sensitivity.

In solid phase microextraction (SPME), a fiber coated with an adsorbent is exposed to the headspace and a very limited amount of the analytes is adsorbed to the fiber. By thermal
desorption, the analytes are then released in the injection port. During static headspace sampling (SHS), a volume (e.g. 1 ml) is sampled and transferred to the injection port. Dynamic headspace sampling (DHS) employs accumulation of compounds in a trapping device filled with an adsorbent material. Hereby, a larger volume is sampled (eg. 1000 ml), typically with replenishment of the sample gas atmosphere by an inert gas. The accumulated compounds are either thermally or chemically (with a solvent) desorbed into the injection port (Ette 2001). DHS was not employed in this project, as it is more complicated to automate and not suited for repeated sampling from a sample. Both SPME and SHS are well-suited for automatisation.

The basis of headspace analysis is the formation of equilibrium, the partition coefficient between the concentration of volatiles in the headspace and the volatiles dissolved in the liquid phase. Hereby, the concentration of the volatiles in the headspace correlates with the concentration in the liquid phase. The equilibrium can be shifted by e.g. temperature, addition of salts and pH changes (Pawliszyn 1997).

For acidic and basic compounds, the equilibrium will be sensitive to pH changes where e.g. acidification, like in LAB batch fermentations, will increase the ratio of protonated versus non-protonated carboxylic acids. In order to do quantitative analysis, it is mandatory to prepare calibration standards in a matrix corresponding to the test samples.

When applicable, HS-SPME has a 10-50x higher sensitivity than SHS (Fabre, Aubry, and Guichard 2002; Pfannkoch and Whitecavage 2000). Headspace sampling with SPME is based on adsorption onto a coated fiber. The fiber coating has a limited capacity which restricts SPME analysis in two aspects. For quantitative purposes, SPME fibers have a limited linearity range and linearity assessments of actual, spiked samples in the expected analytical range are important (Pawliszyn 1999). In the presence of multiple analytes, the analytes with the highest affinity to adsorb will be sampled to the highest degree and may even displace other analytes, termed competitive adsorption and interanalyte displacement (Gorecki, Yu, and Pawliszyn 1999; Semenov, Koziel, and Pawliszyn 2000).

Despite potential concerns regarding the quantitative properties, HS-SPME-GC/MS and even SPME-LC/MS are used for data acquisition for multivariate studies of complex matrices (Serrazanetti et al. 2011; Durante et al. 2006; Vuckovic and Pawliszyn 2011).
The two sampling techniques were complementary to each other. SPME was used for qualitative, semi-quantitative screenings and quantification. In some instances, the headspace concentrations exceeded the linearity range of the SPME fiber and static headspace was used instead. Both methods were applied for fully automated real-time monitoring of the headspace of LAB fermentations.

Static headspace sampling was applied for the quantification of volatiles from LAB fermentations as well as for plugs of agar of LAB ferments (C and CF) collected over the duration of the mold growth test (Example 1G).

5.5.2 Targeted GC analysis of organic acids in LAB ferments
As with the volatiles, short chain carboxylic acids (e.g. formic acid, acetic, propanoic acid) are among the potential major fermentation and antifungal products from LAB and PAB. These compounds were not included in the LC/MS screening protocol. Instead, they were analyzed by GC/MS as their ethyl esters after derivatisation and extraction in diethylether according to an internal DuPont Protocol (applied in Example 1H). The basic principle of the protocol was similar to the method applied by Ryan et al, 2009 for the targeted analysis of 2-hydroxy-3-phenylpropanoic acid in wheat sourdough (Ryan, Dal Bello, Czerny, et al. 2009).

EXAMPLE 6 - DATA PROCESSING

After completion of data acquisition, the next step is the data processing. The scope of data processing is to convert the large quantities of data into interpretable information. The type of available information depends on the biological question and how the answer is planned. The question can be classification of organisms, assessment of an effect of an intervention or, as in this work, to identify biomarkers responsible for the antifungal effect.

Data processing can be subdivided in two subparts, namely data pre-processing and data mining as outlined in figure 6.1 (Boccard, Veuthey, and Rudaz 2010; E. Want and Masson 2011). In short, pre-processing converts the raw analytical data to a format, which is applicable to a range of uni- and multivariate data mining tools. The boundaries between the two subparts of data processing are somewhat fluent. The data processing is an iterative process where e.g. choices of normalization and scaling will affect the data mining. The outcome of data mining may again result in alternative normalization and scaling steps.
In the following sections the focus will be on the considerations and decisions made to search for antifungal compounds by LC/MS data. Although the type of instrumentation affects the steps involved, the overall principles of the workflow could be applied to GC/MS data as well. As mentioned in Example 3, the data processing strategy is necessarily different as a result of the frequent need for pre-analysis derivatisation and the ionization in GC/MS technique. Processing of untargeted GC/MS analyses was only applied to a very limited extent and no further comments will be made regarding this type of data.

6.1 Pre-processing

6.1.1 LC/MS data structure and properties

The overall structure of an LC/MS dataset is outlined in figure 6.2. Each MS spectrum was acquired for the full m/z range defined, e.g. m/z 60 - 1250. During data acquisition, the mass spectra are stored as continuous profiles or as centroided. A centroided spectrum is the result of mass peak apex detection. In comparison to profile spectrum, centroided or line spectra only contain apex positions and intensity, and hence information of peak widths and noise is filtered out. Mass peaks were centroided prior to storage to minimize files sizes and to reduce downstream processing time. Post-run external mass calibration was performed using the spectrum of sodium formate clusters. Through the studies, the mass accuracy of acquired data was better than 2 ppm, which is in accordance with the specifications by the vendor (Honore, Thorsen, and Skov 2013). The mass resolution (full width half maximum at m/z 400) was in the range of 25,000 - 50,000 as earlier demonstrated (Pelander et al. 2011).

For both LC modes (RPC and HILIC), UPLC enabled chromatographic peak widths are in the range 5 - 20 seconds (0.08 - 0.33 minutes). In order to sample sufficient data points to describe a peak, 15 - 32 data points are required (Snyder, Kirkland, and Glajch 1997). MS data was acquired with a scan frequency of 3 s⁻¹ and hence 15 - 60 data points (scans) were available to define a peak.

6.1.2 Feature extraction

The purpose of this critical step of the pre-processing is to convert the collected array of three-dimensional raw LC/MS data files into an organized data matrix suitable for subsequent multivariate data analysis. The resulting matrix contains the intensities (peak height or area) of n samples and k variables or 'features'. Due to the relatively soft ionization by atmospheric pressure interfaces, each 'feature' ideally represents one compound identified by the combination of m/z and retention time, i.e. m/z@rt.
The result of the process is:
- Dimension reduction of the data
- Removal of retention time and m/z inconsistencies by alignment of the data
- Reduction of noise

Numerous commercially and freely available software packages (SWs) are available for supporting this process (E. Want and Masson 2011; Castillo et al. 2011). Examples of commercial SW are ProfileAnalysis (Bruker Daltonics), Sieve (Thermo Fischer) and Markerlynx (Waters).

Open-source, freeware SWs are e.g. XCMS (Smith et al. 2006), Metalign (Lommen 2009) and MZmine2 (Pluskal et al. 2010; Katajamaa, Miettinen, and Oresic 2006), which are often used as benchmark for new solutions (Gurdeniz et al. 2012; Lange et al. 2008; Koh et al. 2010; Wei et al. 2012). The feature extraction is performed by a series of algorithms. The SWs differ in the arrangement and the mode of operation of the algorithms (Castillo et al. 2011; Katajamaa and Oresic 2007).

The feature selection process is described based on the workflow in MZmine2. Some of the steps are illustrated in figure 6.3. The raw MS data was centroided by the data acquisition SW prior to export and MS peak detection could be omitted. Instead, data was filtered for signals below a noise threshold. After filtering, series of extracted ion chromatograms were constructed by connecting consecutive m/z values spanning over multiple scans according to a defined m/z tolerance. The individual chromatograms were then deconvoluted into individual peaks using a peak finder algorithm. The algorithm in general performs well with little noise and well-defined peaks, but fails with poorly resolved peaks like the ones shown in figure 6.3c as shown in recent studies (Wei et al. 2012). After deconvolution, peaks were aligned across the samples.

During the previous steps, some peaks were missed due to low intensity, poor peak shape or sub-optimal parameter settings. These peaks will appear as zeros in the feature table. Presence of zeros instead of the actual low value, may lead to misinterpretations of the data. To minimize presence of zeros, a gap filling algorithm was used to re-examine ion chromatograms and determine the intensities according to the full list of features identified. The number of variables could be reduced slightly by removal of Isotope features. Isotope peaks (primarily A+1) were removed from the peak tables by filtering for peaks, which fulfilled criteria in terms of retention time
and m/z. Tolerances for m/z and retention time (drift, peak width) were acquired from inspection of QC-samples throughout actual sample sequences.

ProfileAnalysis 2.0 and MZmine2 were compared during the project (not published data). In ProfileAnalysis, the feature selection is a one-step-process based on the 'Advanced Bucketing' algorithm (Krug et al. 2008). Parameter settings can be based on manual iterative processing and inspection of the resulting assigned features within individual files. Unfortunately, the SW does not enable the visual inspection of the performance of alignment of features across multiple files. ProfileAnalysis contains excellent filtering options for removing features only present in a limited number samples within defined groups or the full dataset. Being part of the vendor SW assembly, ProfileAnalysis offers seamless connections to other Bruker Daltonics SW tools for visual inspection of chromatograms and analysis of mass spectra. On the other hand, ProfileAnalysis does not contain any algorithm for gap filling.

To achieve optimal feature selection, both the performance of the SW algorithms as well as proper parameter selection are critical. MZmine2 was considered to be superior to ProfileAnalysis due a number of reasons, being:

- Facile interactive parameter optimization by the visualization of e.g. noise level setting, deconvolution and alignment
- Option for gap filling
- Visualization of resulting alignments and corresponding chromatographic profiles

Besides the above-mentioned aspects, some differences were noticed during feature selection. The parameter settings for feature selection in ProfileAnalysis are limited to thresholds for signal-to-noise ratio, correlation coefficient, minimum number of spectra to include and a smoothing factor. In spite of meticulous optimization of these, ProfileAnalysis failed to assign closely eluting constitutional isomer (lie) and a co-eluting stable isotope labeled compound (Leu-\(^{1^b}C_{22}\)) as outlined in figure 6.4.

Inspection of feature tables from two SWs generally revealed significantly higher counts of zero values in ProfileAnalysis compared to MZmine2. This was expected due to the absence of a gap filling algorithm in the latter. In order to further assess the performance of the two SWs, the same dataset (HiLIC in ESI pos of 96 injections of samples from the exponential phase of 4 different types of fermentations, data not published) was subjected to feature extraction with the two SWs.
Furthermore, the same dataset was integrated using a targeted quantification SW, QuantAnalysis (Bruker Daltonics) based on predefined retention times and m/z ratios ± 10 mTh of 15 selected nutrients. The correlation between peak areas normalized with internal standard Phe-D₅ (i.e. A/A₀) of either of the untargeted SWs against the targeted SW showed significant differences as outlined in the examples in figure 6.5. As expected, the ability to assign features decreased with a decrease in relative response (A/A₀). A corresponding increase in the number of zero value features was observed in ProfileAnalysis. In contrast, only one peak assignment in the low end of the Riboflavin response range was missed by the MZmine2 SW in the three examples. Besides the problem of feature assignment, the imprecision of the ProfileAnalysis areas was considerably higher than the corresponding from MZmine.

Based on the relatively soft ionization in AP ESI, each feature should ideally represent one compound. However, this is not always the case due to formation of different adducts, clustering and ion-source fragmentation. As shown for glucose (Glc) in figure 6.6. MZmine2 contains an algorithm for adduct search which enables assignment of adducts based on user-defined mass differences in between adducts, e.g. sodium and proton adducts are 21.9819 Da within a given retention time window. The algorithm provides inspiration for tentative assignment of features, but assignments should be supported by at least a comparison of raw chromatographic profiles and preferably spiked-in standard material to avoid misinterpretations.

To summarize, the process of feature extraction is a very critical part of the metabolomics workflow. Inappropriate selection of parameters and SW may deteriorate an otherwise excellent raw dataset.

6.1.3 Normalization

The aim of metabolomics experiments is to induce biological changes which are expressed in variations in metabolite concentrations. To be able to address the changes of interest, it is important to realize what variations are expected within (exo)metabolomics data. Metabolomics data can be characterized to contain (van den Berg et al. 2006):

1. Unwanted or un-induced biological variations - i.e. substantial variations in concentration under identical biological conditions
2. Analytical or technical variation - sample handling, work-up, analytical errors
3. Differences in orders of magnitude between measured metabolite concentrations, nutrients and signaling molecules
4. Differences in the fold changes due to induced variation. Central/primary metabolism displays minor relative variations whereas metabolites in secondary pathways display larger differences as response to environmental conditions.

5. Heteroscedasticity, i.e. the dissimilarity of variance between variables.

Normalization or row scaling is employed to remove, or reduce unwanted experimental variations or errors in responses (height or area) of the analytical data without removing interesting biological variation. If not removed, the unwanted variations, either as bias and/or imprecision hamper interpretation and validity of the subsequent multivariate data analysis. The source of variations can be categorized as biological and/or analytical. The unwanted biological variation originates from the uncontrolled and unrecognized factors within the biological system, e.g. for microbial metabolomics' inhomogeneous cell growth.

The analytical variation is the combined effect of sample handling, as well as of drift in response during and in-between batches of chemical data acquisition. Continuous shifts in response over time is designated drift and is due to e.g. contamination of analytical column and detector, change in detector sensitivity and change of sample composition (Burton et al. 2008). In between data acquisition of sets of analytical batches, shifts in analytical response occur as the consequence of performance optimizations (column change, maintenance, cleaning tuning).

Metadata, like optical density, cell counts and biomass can serve for normalization of biological variations within treatments, e.g. for ensuring readily comparable specific rates of turnover.

Besides applying metadata, two different normalization strategies can be applied, either based on statistically based optimal scaling factors or standards (E. Want and Masson 2011). Examples of statistical row scaling factors are intensity unit norm (Scholz et al. 2004) or intensity medium (W. Wang et al. 2003).

The use of standards can involve either one or multiple internal standards added during sample preparation (Kooke et al. 2011). The internal standards are selected to represent compound classes and/or regions of interest throughout the chromatogram. A limitation in this aspect is the cost and availability of labeled materials. A costly, but effective way to circumvent limitations in availability is to use fully isotope labeled extracts of organisms (Wu et al. 2005). The use of stable isotope labeled internal standards is well-suited for targeted studies.
In terms of untargeted studies, internal standards should be used with caution. Differences in ionization (ion suppression/enhancement), and peak shapes to the multitude of compounds present in an untargeted study may introduce artifacts in the data, and the effect should be evaluated prior to further analyses, e.g. by inspection of PCA scores versus run order.

Besides serving for quality assurance of the sample acquisition, QC or pooled samples can also serve for normalization. When properly and repeatedly distributed in the analytical sequences, QC-samples can serve both for drift not corrected by internal standard(s), but also for shifts in-between batches (Kloet et al. 2009; Dunn et al. 2011; Kamleh et al. 2012).

Two internal standards were applied during the experiments (Example 1H). Due to the risk of matrix components that could affect the response of either of the two ISs, their ratio versus sample number were inspected for any systematic behavior that could relate to sample type prior to use for normalization. Normalization with either of these resulted typically in a minor improvement of the correction of drift whereby the QC sample replicates clustered more tightly as shown in figure 6.7. The internal standard served for correcting minor variations in the injection volume, as well as minor changes in the detector response due to contamination and drift of the detector.

6.1.4 Scaling

Upon normalization, use of appropriate data pretreatment algorithms enables the focus on specific properties within the data. The choice of pretreatment algorithm depends both on the purpose of the study, but also on the nature of the data e.g. in terms of heteroscedasticity.

Centering or mean centering removes offset in between metabolite levels in order to be able to focus on differences in the data. Scaling may adjust for the differences in fold differences by converting into differences relative to the scaling factor. Selection of the scaling method is a balance between leveling out the importance of variables versus inflation of the relatively large measurement errors of low level metabolites.

Scaling can be performed using the data dispersion (e.g. standard deviation) or by a size measure (e.g. the mean). Transformations are non-linear conversions generally applied to correct for heteroscedasticity and convert multiplicative into additive relations. The transformation reduces larger values relatively more than smaller values and is therefore included as 'pseudo scaling'. A number of centering and scaling methods with their relevance are shown in table 6.1 (van den Berg et al. 2006).
Table 6.1 Centering and scaling methods relevant for metabolomics data (adapted from (van
den Berg et al. 2006))

<table>
<thead>
<tr>
<th>Method</th>
<th>Formula</th>
<th>Purpose</th>
<th>Potential problem</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean centering</td>
<td>$\bar{x}<em>{ij} = x</em>{ij} - \bar{x}_i$</td>
<td>Remove offset and hereby focus on differences in the data</td>
<td></td>
</tr>
<tr>
<td>Autoscaling</td>
<td>$\bar{x}<em>{ij} = \frac{x</em>{ij} - \bar{x}_i}{s_i}$</td>
<td>Make all metabolites equal of importance and compare them based on correlations</td>
<td>Inflation of noise or measurement error</td>
</tr>
<tr>
<td>Range scaling</td>
<td>$\bar{x}<em>{ij} = \frac{x</em>{ij} - \bar{x}<em>i}{(x</em>{i_{max}} - x_{i_{min}})}$</td>
<td>Make all metabolites equal of importance and compare them relative to biological response range</td>
<td>Inflation of noise or measurement error</td>
</tr>
<tr>
<td>Pareto scaling</td>
<td>$\bar{x}<em>{ij} = \frac{x</em>{ij} - \bar{x}_i}{\sqrt{s_i}}$</td>
<td>Reduce the relative importance of large values but to a lesser degree than autoscaling</td>
<td>Sensitive to large fold changes</td>
</tr>
<tr>
<td>Vast scaling</td>
<td>$\bar{x}<em>{ij} = \frac{(x</em>{ij} - \bar{x}_i) \cdot \bar{x}_i}{s_i}$</td>
<td>Based on prior group knowledge, focus on metabolites that show small variations</td>
<td>Not suited for large induced variation</td>
</tr>
<tr>
<td>Level scaling</td>
<td>$\bar{x}<em>{ij} = \frac{x</em>{ij} - \bar{x}_i}{s_i}$</td>
<td>Focus on the relative response</td>
<td>Inflation of noise or measurement error</td>
</tr>
<tr>
<td>Log transformation</td>
<td>$\bar{x}<em>{ij} = \log(x</em>{ij})$</td>
<td>Reduction of heteroscedasticity and converting multiplicative effects to additive</td>
<td>Zeros need to be removed from dataset and challenges with values with relatively large standard deviations</td>
</tr>
<tr>
<td>Power transformation</td>
<td>$\bar{x}<em>{ij} = \sqrt{(x</em>{ij})}$</td>
<td>Reduction of heteroscedasticity without the problems of log transformations</td>
<td></td>
</tr>
</tbody>
</table>

Mean: $\bar{x} = \frac{1}{j} \sum_{j=1}^{j} x_{ij}$; standard deviation: $s_i = \sqrt{\frac{\sum_{j=1}^{j} (x_{ij} - \bar{x}_i)^2}{j-1}}$; $\bar{x}$ and $\bar{x}$ represents data after pretreatment steps.

The standard deviations of the injections of QC samples represented the technical repeatability of the analytical platform. The data was used for assessment of the distribution of variance within the detected variables and hence evaluation of the effect of scaling and transformation.

The effect on heteroscedasticity of Pareto and logarithmic transformation (after an addition of 20 to all feature responses to eliminate zeros) is shown in figure 6.8 as outlined by Masson and coworkers (Masson et al. 2011). The data is RP ESI pos of QC samples and originates from the study detailed in Example 1H. The data was ranked according to mean intensity (area).
The data followed the normal trend of LC/MS data of increase in variability with increasing signal intensities (figure 6.8a+b) as e.g. observed by Masson and coworkers (Masson et al. 2011).

The large variation in standard deviation was reduced by Pareto scaling (figure 6.8c) but by no means as effective as the log transformation (figure 6.8d). Prior to PCA, similar pretreatments were applied to the test samples from the same dataset as applied above (figure 6.9, data from Example 1H). According to the scores plots, the different treatments did not affect the classification of the three LAB strains. The pretreatment did however have a significant impact on the ability to interpret the loadings plots. As expected, the autoscaling inflated the measurement errors and hereby hampered identifying the variables responsible for the clustering. Although with difference in the tightness of clustering of the less important loadings, both Pareto and log transformation displayed similar variables responsible for the classification of the three LAB strains.

6.2 Multivariate data analysis

The data table obtained from the feature extraction and pretreatments is a large matrix X of n observations (e.g. samples) and k variables or features. The number of variables often by far exceeds the number of observations, i.e. k >> n. The features are expected to be highly correlated and most importantly, biological effects are expected to involve more than one feature, potentially multiple features. The multivariate characteristic of the data matrix and the scope for understanding underlying biological mechanisms calls for multivariate data analysis techniques.

Multivariate data analysis techniques allow for finding and visualizing underlying structures in data that are not readily apparent or cannot be correctly evaluated by univariate statistics. The multivariate data analysis can identify trends, outliers, and relationships between observations as well as among variables, which correlates with the scope of this work, to find biomarkers responsible for antifungal effect.

Multivariate methods can, as a broad system, be classified as either unsupervised or supervised. In unsupervised or exploratory methods, the data is analyzed based on a minimum of a priori assumptions, e.g. class membership.

On the other hand, supervised methods aim to relate a set of pre-known or otherwise determined properties to the multivariate dataset. This could enable understanding correlations between dependent and independent variables based on different types of measurements or to build
regression models which can be used for prediction of sample properties. As an example, linking the metabolic footprint to inhibitory effect could possibly enable the prediction of antifungal effect of a sample based on measurement of selected compounds.

Among the wealth of methods found within the field of multivariate analysis, only two were applied herein. This includes the unsupervised principal component analysis (PCA) and supervised partial least squares regression (PLS). Although more advanced methods have not been applied, it is worthwhile to mention examples with special properties that correspond to experimental design and properties of the resulting data (Hendriks et al. 2011; Boccard, Veuthey, and Rudaz 2010).

Among these are Analysis of Variance (ANOVA)-Simultaneous Component Analysis (ASCA), which has been reported well-suited for finding underlying structures in data acquired according to an experimental design, e.g. factorial designs and time-resolved measurements (Smilde et al. 2005). In order to explore the full information embedded in multi-way data, methods based on Parallel Factor Analysis (PARAFAC) or PARAFAC2 can also be applied (Bro 1997), but due to the low-rank nature of these techniques they are limited to subparts of the data (Skov and Engelsen 2013).

6.2.1 Principal Component Analysis

Principal component analysis (PCA, (Hotelling 1933)) is an example of an unsupervised multivariate method. PCA is a projection method that transforms the multidimensional data table into a lower dimensional space that approximates all rows, e.g. samples in the matrix $X$. PCA is a decomposition of $X$ into a linear sum of vector products, $tp$. The first principal model component, PC1 ($t_1p_1$) describes the largest variation in the data matrix and is oriented along the axis of largest variation (also the direction with best least squares fit). The loading vector $p$ (k variables) defines the new direction in the original variable space. For example, important variables are given a higher load(ing). The projection of the samples onto the loadings vector yields the scores vector $t$ ($n$ observations). The second model component is orthogonal to the first component and spans the second largest variation, etc. For $i$ components, the PCA model can be formulated as

$$X = TP^T + E = t_1p_1 + t_2p_2 + \ldots + t_ip_i + \epsilon \quad i = 1, 2, \ldots$$

where $t_i$ is the scores vector of the $i$ principal component and $p_i$ is the loadings vector of the $i$ principal component. The part of variation not described by the model forms the residuals $\epsilon$. The scores represent the coordinates of the observations (samples) in the PCA model. Hence, the sample patterns can be visualized by e.g. scatter plots of scores (plotting e.g. $t_2$ vs. $t_1$). The
loadings define the contribution of the original variables to build the scores. A scatter plot of loadings will show the influence of the individual X-variables (e.g. features) in the model. Positions in the score plot correspond to the directions in the equivalent loadings plot and these two plots must always be interpreted together.

Consequently, the loadings plot will identify which variables (i.e. features) that separate groups of observations (e.g.) samples. PCA serves different purposes in metabolic footprinting and was applied on different levels of the workflow (data from Example 1H).

Passing the basic inspection of raw data files as mentioned in Example 5, the next level of data quality control was performed by PCA after feature extraction. The metabolic footprint of biological replicates of three different Lb. paracasei strains was analyzed by RP ESI neg resulting in a matrix of 70 observations with 142 features (or variables). An initial control is based on scores plot of the first two PCs of all acquired data (without sample blanks and QC-old). The PCA scores plot showed a centered and tighter clustering of the QC samples than of the test samples (fig 6.10a). Two positive indications were drawn from this. The samples were stable in the auto sample (at 5°C) throughout the injection sequence. The technical imprecision was less than the variation in the samples. Depending on the system and the properties of the samples, the equilibration injections may show a trajectory or offset, which is acceptable. Absence of run order effects was ruled out by inspection of the first two-three PCs of the scores versus run number as no trends could be observed (6.10b-c). Run order effects visible in PC1, PC2, or PC3 would have invalidated the dataset for further analysis if they could not have been significantly reduced by normalization.

At this stage, it was valuable to notice that the explained variances of the first two principal components were more than 70% (54% + 18%) of the variation in the data, and hence the dataset was relevant for further data mining.

Additionally, PCA served to visualize relationships between observations and identification of patterns of association among variables. The metadata showed that two of the strains, LAB B and C produced significantly higher biomass than LAB A and C. Additionally, LAB B and C strains acidified the medium to a lower pH. The Pareto-scaled PCA model scores plot of PC2 versus PC1 readily shows a grouping in accordance with the metadata along PC1 (figure 6.1 1a). The corresponding loadings plot shows that the nutrients, especially glucose (Glc) correlates negatively with PC1, whereas the formed acids including lactic acid (La) correlate positively with PC1 (fig 6.1 1b).
According to the scores plot, the LAB B and D differed in the PC2 axis. The explanation for the difference in the two strains is again found in the loadings plot, which shows that the ferment of LAB B contained relatively higher amounts of the feature m/z@rt 251.08@2.00 as well as aromatic 2-hydroxy acids (OH-Ind-Pr, OH-(OH-Phe-Pr) and OH-Phe-Pr). Conversely, LAB C contained relatively more of branched 2-hydroxy acids (OH-Me-Pe).

6.2.2 Partial Least Squares Regression

Partial least squares regression (PLSR) enables the relation of a set of potentially collinear independent variables X (here, features) to one or more dependent variables, Y. The ability to handle numerous, noisy and correlated variables makes PLS a valuable tool (Wold and Sjostrom 2001). PLS is a supervised method and can be used with continuous data or categorical data (e.g. class information), whereby the approach is designated partial least squares regression (PLSR) or discriminant analysis (PLSDA), respectively. PLS, like PCA, is a projection method based on decomposition into orthogonal components. In PCA, the decomposition is performed to explain the maximum variance in X. In PLS, both blocks (X and Y) are decomposed simultaneously to obtain a maximum explanation of variance in X as well as Y. The PLS regression model can be written as

\[ Y = X \mathbf{B} + \mathbf{F} \]

where \( \mathbf{F} \) is the residuals and \( \mathbf{B} \) is the PLS regression vector(s) determined during the model building.

For many purposes, PLS serves to predict Y based on X variables. As PLSR generates a low dimensional subspace while using the additional Y information to include the Y-related information in X, the process results in enhanced focus on variables in X correlated to Y. This ability has made PLS a central tool in metabolomics studies. For typical two-class problems e.g. with the comparison of control versus treatment, PLSDA is a highly used tool for biomarker discovery (e.g. (Bijlsma et al. 2006; Dixon et al. 2007)). Likewise, PLSR is applied for continuous dependent variables, e.g. for microbial metabolomics (Braaksma et al. 2011). Translated into the terminology used herein, PLS models based on biological activity as \( \mathbf{y} \) should emphasize the features (representing compounds) that when put together (or summed/added) in different weights are highly correlated with the antifungal effect.

6.2.3 Validation
With metabolomics data where the number of variables typically exceeds the number of observations, there is a risk of overfitting. Hence, careful validation of the models obtained is critical. Absence of model validation will readily lead to erroneous conclusions, e.g. like PLSDA classification based on un-validated scores plots (Kjeldahl and Bro 2010). Validation serves to determine the optimal number of components, provide estimates of the prediction error and inspection for outliers.

Validation is performed on a set of data with a sufficient number of samples that allows the set to be split into three parts, being calibration (training), validation and test set. The calibration set is used for building the model and the validation set is used for testing prediction. To optimize the use of available data, cross validation is often used. In cross validation, the observations serve both as calibration and validation sets. Repeatedly, alternative segments of the dataset are excluded from the range used for training and applied for validation for a range of models with 1 to N number of models. The segments can be either only one observation or numerous, e.g. randomly selected. Cross validation (CV) serves to determine the number of components, but as the model building and validation are made on the same set of observations, models have been shown to be too optimistic in predictive power. After having selected model parameters, the actual predictive power, and hence the validity of the model should preferably be assessed using the third subset of the data, the test set.

In PLSR, the predictive power can be expressed as the root mean squared error of prediction (based on the test set), RMSEP. In the situation of a too limited sample set to allow for a test set, the root mean squared error of cross validation (RMSECV) has to serve as an alternative for selection as illustrated below (figure 6.12), where four components were chosen.

### 6.2.4 Variable selection

The aim of building the PLS model is to assess the relevance of X variables with regard to the biological activity. Selection of these variables should assist in the interpretation of the biological system. It is important to notice that data pretreatments (as exemplified in section 6.1.4) will have a strong impact on the model and, hence, the variable selection.

Variables with low relevance are easily identified by low loadings in all components as well as variables with low regression coefficients (the components of the regression vector). Accordingly, high loadings and regression coefficients will be indicative of important variables.
Different PLS model measures are available for selecting features strongest correlated with the biological effect, the biomarkers. Variable importance to the projection (VIP) is a combined measure of how much a variable contributes in describing the dependent variable Y and how important that information is for the modeling of the dependent variable (Chong and Jun 2005). VIP scores below 1 indicate non-importance for the model and conversely, the higher values, the higher importance. Based on the algorithm for calculation of the VIP scores, the orientation correlation with scores is lost. In order to interpret whether a feature correlated positively or negatively with the dependent variable, mold growth, the variable selection should be supported by inspection of regression coefficients as outlined in figure 6.13 or univariate correlations studies (Pearson correlation coefficient). In Example 1H, VIP scores were used for ranking the importance and the metabolites, whereas the regression vector served to classify the metabolites as either positively or negatively correlated with mold growth.

The selectivity ratio (SR) is defined as the ratio between the explained variance of each variable and the corresponding residual variance, and hence a high ratio is an indication of good prediction capability (Rajalahti et al. 2009). Except for glucose (glc) and threonine (Thr), none of the features that ranked highest in the two other model measures was recognized of importance according to SR. The discrepancy between SR and the two other variable selection methods was interesting, but could not be readily explained. The three methods may serve to confirm each other, but also to come with candidates to the list of potential biomarkers.

With the extremely high number of variables (in figures 6.9 and 6.10, n = 11, k = 1119) compared to the number of observations, there is a large risk of overfitting and thereby selection of false positives for biomarkers (Broadhurst and Kell 2006). One measure to reduce the risk is the inclusion of more observations to enable a more thorough validation, using either real test set, cross model validation or permutation tests.

Some reduction in the number of variables could be achieved by a more dedicated combination of adducts and clusters originating from the same metabolite e.g. as for Glc. However, information could be embedded in the relative ratio of the adducts and clusters which could otherwise support the understanding of the underlying causal pattern (Andersen and Bro 2010).

Another approach to reduce the number of variables is to employ the features obtained from the QC pooled sample. The strategies include to filter out features with a relative standard deviation (CV%) above a certain threshold, another to exclude features if not present in a given ratio of the
replicate QC injections and finally, the CV% of the features after variable selection (in the QC sample) could serve for validating biomarkers (E. J. Want et al. 2010). A related strategy for focusing only on analytically sound metabolites is to use dilution series of e.g. the QC pooled sample and use the linear correlation as criteria filter (Jankevics et al. 2012).

6.3 Identification
A challenging next step in the metabolomics workflow is to identify the selected variables, the features or biomarkers. Identification in metabolomics terminology is to assign a chemical structure to one or several features. The identification is generally perceived as the bottleneck of metabolomics (Kind and Fiehn 2010).

The challenge embedded in the identification relies on whether the metabolite is previously undiscovered or is non-novel. Full de-novo identification of a previously undiscovered metabolite will require the full regime of spectroscopic (e.g. NMR, IR) and spectrometric (MS) analytical tools. For non-novel compounds the level of certainty in terms of the identification of a metabolite was according to the proposed minimum reporting standards outlined (Sumner et al. 2007):

<table>
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<th>Table 6.2 Levels of certainty for metabolite identification and minimum reporting standards (adapted from (Sumner et al. 2007))</th>
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<td>Level</td>
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In the ESI feature tables, several compounds e.g. glucose (Glc) are represented by a number of features. To cope with this phenomenon, the terminology was expanded by designating adducts and clusters with the compound name, e.g. [Glc+Na]+, [Glc+K]+ as ‘Glc’ and in-source fragments ‘Glc’*. Unknown compound features were designated by m/z and retention time in minutes, e.g. 251.08@2.00.

6.3.1 LC/MS identification
The acquired data was characterized by the capability of the UPLC/HR-MS instrumentation. The UPLC provided reproducible retention, which allowed for using retention as correlation measure from batch to batch, e.g. for comparing peak spectra between ESI pos and neg. The combination of high resolution, high mass accuracy as well as accurate isotope ratios of the MS enabled accurate mass search strategies.

The applied experimental strategy allowed the construction of an internal database of constituents of the chemically defined media and a limited number of expected metabolites with actual retention time for the chromatographic methods and observed ions in both MS modes. Furthermore, literature provided a list of reported exometabolites (Example 2) which was included with name and elementary composition. The database was supplemented with a named list of theoretically derived metabolites based on enzymatic conversion pathways of lactic acid bacteria (e.g. (Liu et al. 2008; Kilstrup et al. 2005). Finally, intracellular metabolites were included from studies of the endometabolome (e.g. (Buscher et al. 2009; Luo et al. 2007; Preinerstorfer et al. 2010)).

The database was used for dereplication of selected raw data files, typically the QC pooled sample and representatives of each treatment. The dereplication provided recognition of known elementary compositions from the database using vendor software algorithm (TargetAnalysis, Bruker Daltonics). The recognition was based on criteria of exact m/z, isotope ratios and retention time (Kolmonen et al. 2007; Ojanpera et al. 2006).

An estimated elementary composition was manually assigned to remaining non-assigned features when feasible. After having assigned the charge and adducts state of the ion, the elementary composition was estimated based on accurate mass and isotope ratio. Elementary compositions and accurate masses were used for queries in the databases such as Kyoto Encyclopediа of Genes and Genomes (KEGG), BioCyc and Human Metabolome Database (HMDB) (Kanehisa and Goto 2000; Caspi et al. 2010; Wishart et al. 2009; Wishart et al. 2013; Wishart et al. 2007).

When feasible, standards for tentatively assigned compounds were used for verifying identity based on relative retention and mass spectrum. The composition of sample blanks served as a negative control to verify that compounds observed did not originate from sample handling and analytical protocols.

6.4 Biological interpretation
The final step of the metabolomic workflow is the biological interpretation of the chemical observations made. The biological interpretation covers a theoretical assessment of whether the metabolites relate to known pathways (e.g. BioCyc and literature) or functionality. Placing the metabolite in a pathway context will enable the search for related precursors or reaction products among the other features present. Correlated dynamic changes in concentrations of e.g. a precursor and the metabolite may support the biological relevance of the metabolite.

However, all interpretations are based on correlations, which may guide towards understanding of the underlying biological effect, but do not imply the actual casual mechanism. In terms of the quest for antifungal tests, the next step was to test whether the identified putative antifungal compounds actually possessed an antagonistic effect against molds at the concentrations produced.

Hence, targeted analysis was applied to determine the concentrations of the antifungal compounds which were then tested for MIC solely or in combinations.

The routes for finding compounds of interest, putative biological pathways and their antifungal effect are discussed in Example 7.

**EXAMPLE 7 - GETTING CLOSER TO REVEALING THE ANTIFUNGAL EFFECT - FINDINGS, IDEAS AND PROSPECTIVES**

The central dogma for the experimental work was to correlate the mold growth on cell-free ferments (CF, the exometabolome) to the corresponding chemical profile. Fermentations should be performed with the chemically defined interaction medium (CDIM) as substrate to increase the probability for discovering new antifungal exometabolites.

Prior to any chemical measurement, it was critical to demonstrate a) that the CDIM supported growth of all involved organisms (bacteria and fungi) and b) similar biological responses as in milk could be observed. Details and abbreviations for the microorganisms included in the studies are listed in table 4.2, section 4.2. Similar biological response was demonstrated in between CDIM and milk, i.e. inhibition of mold on ferments from LAB A (*Lactobacillus paracasei* strain from the co-culture with the antifungal properties) with and without PAB A (*Propionibacterium freudenreichii* subsp. *freudenreichii* strain from the co-culture with antifungal properties) (Example 1F).
7.1 The effect of LAB A cell presence and volatiles (Example 1G)

Inhibition of mold growth was clearly linked to bacterial cell presence, and the effect was removed with the bacterial cells. The inhibition of mold on LAB A CFs corresponded to un-inoculated, but acidified CDIM. The observation led to a range of working hypotheses on the prerequisites for inhibition of mold, including:

a) Cell-to-cell contact could be needed for two reasons. An altered metabolism of LAB would be triggered by sensing the presence of mold. Hereby, the altered metabolism should result in production of antifungal compounds. Alternatively, the antifungal effect could rely on direct, physical contact between the mold and the bacteria.

b) The inhibitory compounds are removed together with the bacterial cells. The bacterial cells were removed by centrifugation and filtration. Compounds with affinity to e.g. the filter surface would be depleted from the filtrate. Alternatively, compounds adhering to the bacterial cell surface would follow the cells.

c) Production of the inhibitory bacterial metabolites was initiated by the physical change of the medium, i.e. from a liquid to a solid. The changes in the diffusion rate could affect availability of the nutrients for the bacteria.

d) Bacterial cells deplete essential nutrients by continued growth in solid medium.

All attempts to enhance additional inhibitory effect into the CF were unsuccessful. As an example, microbial tests showed that presence of mold hyphae during bacterial fermentations did not enhance the inhibitory effect (data not included). Indeed, all observations conflicted with the working strategy of being able to find the compounds in a spent, cell-free medium.

One simple experiment provided an observation to reconsider the above mentioned and generate new hypotheses. Efficient inhibition of mold was demonstrated without any physical contact between a bacterial ferment with cells and CDIM inoculated with mold, only mediated via the shared headspace. Either depletion of oxygen and/or formation of inhibitory volatiles could be responsible for the effect. In line with hypothesis b), the steps for removal of bacterial cells (centrifugation and filtration) and preparation of plates (heating to 48°C and mixing with agar) for the inhibition test could be the cause for loss of volatiles, which again results in limited inhibition effect for the CF.

The formation of volatiles during bacterial fermentations was monitored by headspace analysis (GC/MS). Only two major products, 2,3-butadione (diacetyl) and 3-hydroxy-2-butanone (acetoin)
were detected in the headspace of bacterial fermentations in CDIM (diacetyl profile shown in figure 7.1.a). Plugs from the agar were sampled throughout the mold assay test duration in order to monitor the difference in volatiles after pouring plates. Concentrations of both compounds were measured in the agar plugs (diacetyl profile shown in figure 7.1 b).

The level of diacetyl and acetoin were significantly reduced in agar plates based on CFs compared to the corresponding ones with cells. The difference in inhibition between LAB A cell-containing (C) and CF correlated with the relative concentration difference between LAB A C and CF, i.e. mold growth was significantly reduced on plates with high diacetyl content (figure 7.1 c+d - see also Fig 1G.3).

The antifungal effect of diacetyl and acetoin was tested by spiking these into CDIM (both neutral and acidified). Only diacetyl had significant antifungal effect within the concentration range observed in the agar and both in neutral and acidified media. Hence, diacetyl was a major contributor to the antifungal effect observed for LAB A fermented in CDIM.

By the removal of cells and preparation of agar plates, diacetyl concentration was reduced from ca. 20 to ca. 10 mg/L (figure 7.1). According to the monitoring of the headspace, diacetyl production would have continued and potentially have led to a concentration of approximately 50 mg/L, which may have enabled detection of an inhibitory effect in the CFs compared to the acidified reference plates.

Diacetyl is a well-known metabolite from LAB and is produced chemically by an oxidative decarboxylation of alpha-acetolactate (AL) synthesized from pyruvate as outlined in figure 7.2. So alpha-acetolactate decarboxylase (ALDC) and acetoin-diacetyl reductase (AR) could reduce the content of diacetyl and hereby provide a negative control. Ferments with added ALDC displayed a reduced inhibition effect compared to controls without the enzymes.

Activation of the diacetyl/acetoin pathway in LAB is a response to excessive accumulation of pyruvate within in the cell, and the routing via acetolactate (AL) to acetoin ensures pH homeostasis without the cost of NADH (Tsau, Guffanti, and Montville 1992). Maximum production of diacetyl is observed in the late exponential or at the early stationary phase (Branen and Keenan 1971). Formation of diacetyl has mostly been associated with LAB where during citrate metabolism there is an accumulation of AL, which in the presence of oxygen forms diacetyl (Beresford 2011).
AL also serves as precursor for the synthesis of branched amino acid but with presence of Leucine, no AL is routed for the biosynthesis (Renault et al. 1997). The production of diacetyl in favor of acetoin could be a consequence of acetolactate decarboxylase (ALDC) deficiency and aerobic conditions (Hugenholtz et al. 2000). AL is a labile compound which readily converts to diacetyl in the presence of oxygen and under acidic conditions (Richelieu, Houlberg, and Nielsen 1997; Veringa, Verburg, and Stadhouders 1984). With the present experiments, no distinction can therefore be made whether diacetyl or AL was secreted by the cells (and the AL then converted to diacetyl). The reduction of inhibitory effect by adding ALDC could indicate that AL is secreted from the cell. Secretion of AL could be the consequence of an extended overflow mechanism as recently described for other intracellular metabolites. The extended overflow mechanism results in secretion of intracellular intermediates during conditions with nutrient excess, and as the nutrients become limited there is a re-uptake of the intermediates (Paczia et al. 2012).

Already in 1941, diacetyl was recognized as a fungicidal component effective against molds at concentrations above 86 mg/L (Lagoni 1941) and further supported by several studies, at higher concentrations, though (Rama Devi and Polasa 1985; Jay 1982; Jay, Rivers, and Biosvert 1983). Diacetyl has potent flavor properties and the dose needed to obtain an antifungal effect is considered above an acceptable taste threshold. LAB strains known as diacetyl producers (Streptococcus lactis subsp. diacetylactis) are associated with antimicrobial and antifungal properties but no causal link to diacetyl has been documented (Reddy and Ranganathan 1983; V.K., Grover, and Lai 1989).

The mechanism for antifungal effect is not well-described. A possible explanation could be based on the reactivity of diacetyl. Diacetyl reacts selectively with the guanidine residue of arginine in enzyme and hereby causes a change in the functionality of the enzyme (Riordan 1979). The reaction product was described by Mathews (Mathews et al. 2010). Hence, the antifungal effect of diacetyl could originate from modifications of enzymes within the cell or the cell membrane of mold.

A corresponding antifungal co-culture of Lactobacillus paracasei and Propionibacterium jensenii was thoroughly investigated by Schwenninger at al., both to discover the optimal combination of the two species as well as the compounds responsible for the effect. According to these studies, strong antifungal effect is only present when cells are present and grown in a solid or semisolid medium. No inhibition is observed in CFs prepared by traditional liquid state batch fermentation. Based on bioassay-guided fractionation, several acids were reported to have antifungal effect when compared to non-acidified controls (Schwenninger et al. 2008; Schwenninger and Meile
2004). Although the studies in this project were on mold and not yeast, the absence of activity in CFs was in line with their studies. Not identifying diacetyl in their studies may result from a general focus on the CF and loss of diacetyl during the extensive bioassay guided fractionation.

In order to validate the findings in the model system, experiments were made to determine the formation of volatiles in the food system. Yoghurt fermentations were made with and without LAB A while monitoring the headspace. The headspace of both types contained numerous compounds which increased over time. The relative amount of diacetyl was higher in yoghurt fermentations with the LAB A than without as outlined in figure 7.3.

The addition of diacetyl to milk chemically acidified to pH 4.5 and yoghurt induced a higher inhibition of mold growth than in similar matrices without added diacetyl (figure 7.4).

Unfortunately, all these observations only provide circumstantial evidence for diacetyl being a major contributing factor to the inhibitory effect of the LAB A in yoghurt. Actual concentrations of diacetyl formed in yoghurt with and without LAB A remain to be measured. In case diacetyl proves to be a major contributing factor to the inhibitory effect, this by no means rules out a contribution from other factors.

All in all, it was interesting that the observed effect in CDIM did not come from an unknown exometabolite as hypothesized initially (hypothesis 1, section 1.3), but actually from one already attributed with antifungal properties. A major part of the antifungal effect apparently originated from the relatively larger tendency of LAB A to produce diacetyl (or alpha-acetolactate) compared to the other LAB strains applied for this fresh fermented food system. The production of diacetyl occurred both in liquid and solid CDIM media and without presence of any fungal organisms, although it cannot be ruled out that the LAB A metabolism would be altered by the presence of fungi. This was not as hypothesized in hypothesis 2. However, the conversion of AL to diacetyl requires oxygen (or an oxidizing agent) and proceeds faster under acidic conditions; two properties, which will be affected by the activity of other LABs present in a milk-based system due to their ability to consume oxygen and contribute to acidification.

7.2 The effect of propionic acid bacteria (non-published data)
The observations in the previous section related to the properties of LAB A without the presence of PAB A. But the co-culture also contains PAB A, although a significant part of the antifungal effect was produced by LAB A. This raises the question:
Which role does the propionic acid bacteria (PAB A) play?

The co-culture consists of a blend of the LAB A and PAB, with the main component being LAB A. In terms of antifungal properties, each of the individual strains is outperformed by the combination similarly as shown in food matrices in previous studies (Schwenninger and Meile 2004; El-Shafei et al. 2008; Suomalainen and Mayra-Makin 1999). According to Schwenninger and coworkers (2004): "...The main antifungal features seemed thus to be associated with lactobacilli and were supported by synergistic activities of propionibacteria..." (Schwenninger and Meile 2004).

A time course study was conducted with the two involved strains alone and in combination (LAB A, PAB A and LAB A+PAB A in the ratio 20:1). The fermentations were sampled before, during and after the exponential phase. The samples were analyzed for cell counts, pH and CFs for mold growth and chemical profiles by GC/MS and LC/MS (methods as in Examples 1G and 1H). In the LAB A+PAB A combination, the slow-growing PAB only maintained a constant number of live cells as added during inoculation. Cell counts for LAB A and acidification curves were not significantly different for LAB A + PAB A and LAB A. Based on the chemical profiles of the CFs by LC/MS and GC/MS, the only difference was an increased amount of diacetyl in the combined LAB A +PAB A in one sample point close to the end of the exponential phase. Otherwise, no difference between LAB A and LAB A + PAB A could be observed by unsupervised PCA of the data. In both fermentations, presence of 2-hydroxy branched and aromatic acids was detected from the end of the exponential phase and onwards. Most importantly, the mold inhibition was not significantly different between the LAB A and the LAB A + PAB A CFs.

Instead of testing the bacteria growing simultaneous as a co-culture, fermentations of bacteria species were made with pre-fermentations of the other species. Pre-fermentation of PAB A (4 days in CDIM) prior to fermenting LAB A resulted in an induction of LAB A growth observed by OD.

Presence of 1%v/v PAB A ferment further enhanced LAB A (designated LAB A+) cell growth and with the enhanced growth followed increased production of diacetyl as illustrated in figure 7.5. With the presence of PAB A C, the production of diacetyl accelerated towards the end of the exponential phase.
Although the difference in diacetyl concentration between LAB A and A+ was reduced in agar plates (figure 7.5 b), mold growth on LAB A+ was inhibited to a larger degree, delaying their outgrowth by one day.

Only a negligible difference was observed between the mold growth on CF and REF (acidified CDIM medium) as seen in figure 7.1 c+d). Likewise, no significant difference between the mold growth on CFs of LAB A and A+ mold inhibition could be observed (data not shown).

Hence, in terms of discovering compounds responsible for the antifungal effect, the CFs seems to be less interesting. However, metabolic footprinting by LC/MS of the CF from LAB A and LAB A+ could assist in describing the metabolic changes induced by PAB C on the LAB A. According to unsupervised PCA, the major difference was a relatively larger consumption of nutrients (glucose, adenine, adenosine and amino acids) and an unassigned intermediate and formation of 2-hydroxy-C6-acid (OH-Me-Pe) by LAB A+ (figure 7.6). Based on univariate statistics, none of the observed changes were significant.

In accordance with previous studies (Schwenninger et al. 2008), two aromatic 2-hydroxy acids (phenyllactic acid and (4-hydroxyphenyl)lactic acid) were detected in the LAB A CF. Additional four, 2-hydroxy acids were identified. The 2-hydroxy acids will be discussed and listed (table 7.1) in the following section.

All in all, the role of PAB A presence was an enhancement of LAB A growth when studied in CDIM. The increased growth then resulted in a larger consumption of nutrients, and furthermore in increased production of antifungal metabolites like diacetyl and 2-hydroxy acids. The combined effect of these factors resulted in a larger degree of inhibition. One could speculate, whether the presence of PAB A in a yoghurt system provides a selective enhancement of the LAB A growth over other LAB species.

7.3 Beyond the volatiles - comparative study of Lb. paracasei strains (Example 1H)

Diacetyl contributed to the antifungal properties of the LAB A strain by delaying the outgrowth of the mold. However, the effect could be supported by the production of other metabolites, when the bacteria continue to grow in the agar plates. The two indicator molds differed in their sensitivity toward inhibitory factors with Penicillium soiitum DCS 302 being most sensitive towards low pH (or high lactic acid, Example 1J) and Penicillium sp. DCS 1541 most sensitive towards diacetyl (see figure 7.1).
To search for compounds with effect(s) beyond diacetyl and formed later in the fermentation, a range of *Lb. paracasei* strains were screened for antifungal effect in the CF. Based on their different inhibitory effect of their CF, three *Lb. paracasei* strains (listed according to mold growth: LAB A > LAB B > LAB C) were selected for trials. All strains were diacetyl producing and to reduce the effect of the diacetyl, cells were removed prior to analysis. Only a slight difference in mold growth could be observed during the initial phase on the CF from each of the three LABs and the acidified reference. This was observed as a variation in number of white pixels, which corresponded to the pH (lactic acid) sensitivity of *Penicillium solitum* DCS 302. The absence of any difference in growth of *Penicillium* sp. DCS 1541 supported that the effect of diacetyl had been efficiently reduced.

The largest difference between the three LAB CFs and acidified reference was observed on day 5 of the mold incubation period, but the same relative trend was observed already from day 3.

Unsupervised multivariate data analysis (PCA) of the chemical profiling data of the three ferments demonstrated that they could be grouped in accordance with the biological metadata obtained. Comparing the three strains, two of the strains (LAB B and C) provided a higher degree of acidification and higher biomass. Correspondingly, LAB B and C produced higher amounts of acidic metabolites and consumed a larger amount of nutrients.

Altogether, the CFs of the three LAB strains created a variation in the media for mold growth in terms of the relative amounts of formed metabolites and remaining nutrients. Using PLSR, the footprints were correlated to mold growth given as the sum of white and green of pixels (on day 3). The scope was not to build a model for prediction of mold growth based on metabolic footprints but to investigate what compounds correlated with mold growth in terms of consumed and formed metabolites.

Consumption of glucose, amino acids (Phe, Leu+Ile, Trp, Met, Tyr, Val), adenosine and adenine was found to be oppositely correlated to mold growth. Besides glucose (and Gin), none of the nutrients were depleted in the most inhibiting strain, LAB B. Absence of glucose was shown to have a negative effect on mold growth.

The three LAB strains could be distinguished by their production of 2-hydroxy acids. LAB A and C produced relatively higher amounts of branched chain 2- hydroxy acids, whereas LAB B produced relatively higher amounts of aromatic and sulfur containing 2-hydroxy acids. The production of the
respective classes of acids correlated inversely with the consumption of Met, branched chain and aromatic amino acids, which indicates the mentioned amino acids to be precursors for the 2-hydroxy acids as listed in table 7.1.

Table 7.1 Systematic, trivial names, structures and proposed precursor for 2-hydroxy acids identified

<table>
<thead>
<tr>
<th>Systematic name (abbreviation)</th>
<th>Trivial name(s)</th>
<th>Structure</th>
<th>Proposed precursors</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-hydroxy-3-methylbutanoic acid</td>
<td>2-hydroxy-isovaleric acid</td>
<td></td>
<td>Val</td>
</tr>
<tr>
<td>2-hydroxy-4-methylpentanoic acid (OH-Me-Pe)</td>
<td>2-hydroxyisocaproic acid</td>
<td></td>
<td>Leu (Leu+Ile)</td>
</tr>
<tr>
<td>2-hydroxy-3-phenylpropanoic acid (OH-Phe-Pr)</td>
<td>Phenyllactic acid</td>
<td></td>
<td>Phe</td>
</tr>
<tr>
<td>2-hydroxy-3-(4-hydroxyphenyl)propanoic acid</td>
<td>(4-hydroxyphenyl)lactic acid Or Hydroxyphenyllactic acid</td>
<td></td>
<td>Tyr</td>
</tr>
<tr>
<td>2-hydroxy-3-(1H-indol-3-yl)propanoic acid</td>
<td>Indolelactic acid</td>
<td></td>
<td>Trp</td>
</tr>
<tr>
<td>2-hydroxy-(4-methylthio)butanoic acid</td>
<td></td>
<td></td>
<td>Met</td>
</tr>
</tbody>
</table>

a) OH-Me-Pe represented the combined feature from 3- and 4-methyl acid isomers, i.e. from lle and Leu, respectively

b) Not previously described in relation to cultures with antifungal properties

The inhibitory effect of acids individually and in combination was tested by addition to an acidified media.
All six identified acids possessed antifungal effect, but as previously observed (Example 2), none of them accounted on their own for the effect observed. Three of the six acids identified (from Val, Trp and Met) had not been described in relation to antifungal cultures.

The formation of the 2-hydroxy acids is assumed to originate from the transamination route as outlined in figure 7.7. Specific aminotransferases enable the exchange of an amino group with a keto group from a 2-keto acid (here oxoglutaric acid). The enzymes are selective towards the classes of amino acids according to the names, i.e. branched chain aminotransferase (BcAT) acting on Leu, Ile, Val and methionine whereas aromatic aminotransferase (ArAT) acts on Phe, Tyr, Trp. The formed keto-acids are then reduced to the hydroxy acids by different hydroxy acid dehydrogenases (Liu et al. 2008).

In terms of flavor development, the route with decarboxylation is more favorable. The formation of hydroxy acids is considered favorable compared to the flavor forming pathway under conditions with the presence of suitable amounts of an electron acceptor, e.g. O_2 (Chambellon et al. 2009). The transamination reaction is described for Lactococcus lactis to serve as an additional mechanism to reduce toxic stress from other 2-keto acids, i.e. oxaloacetic acid and pyruvate resulting in a production of 2-hydroxy acids. (Pudlik and Lolkema 2012).

The detection of and the relative difference in hydroxy acids could hereby provide interesting information about the different LABs.

- The relative amounts of the 2-hydroxy acids could be descriptive of which aminotransferase (BcAT/ArAT) is expressed and active in each strain
- None of the keto-acids corresponding to the identified hydroxy acids were detected. The absence of the keto-acids indicates a reductive environment, i.e. surplus of NADH
- Presence of the hydroxy acids are indicative of the presence of hydroxy dehydrogenases

The excess of amino acids in the CDIM may serve as an additional way of reducing the intracellular excess of pyruvate by the transamination. Hereby pyruvate is transformed into alanine and the keto/hydroxy acids are formed. This correlates with the observed combined induction of both diacetyl and 2-hydroxy acids by the addition of PABA.
The transamination is a general trait of LAB but the genetic and regulated differences in the downstream pathways (for either flavor compounds or 2-hydroxy acids) could indicate whether the bacterium has flavor or protection abilities.

In conclusion, the antifungal abilities of the *Lb. paracasei* strains examined are believed to be embedded in the stress response mechanism related to maintaining pH homeostasis in situations where excess of intracellular pyruvate is produced. To cope with the surplus, pyruvate is believed to be routed to larger extent via the acetoin/diacetyl pathway. In the *Lb. paracasei* examined, the activity of ALDC is insufficient to convert the amounts of routed alpha-acetolactate. The insufficiency may be attributed to either absence of genes for ALDC, limited expression or the down regulation of the enzyme. The formation of hydroxy acids may then be a complementary mechanism to further reduce the excess pyruvate not relieved via the acetoin/diacetyl pathway. Increasing amounts of 2-hydroxy acids is then considered more to be a symptom of the deficiency to handle excess pyruvate (via the acetoin diacetyl pathway) rather than being the causal link.

The use of a model system simplified the subsequent chemical analysis and enabled the investigation of the dynamic changes induced by the bacteria. In spite of the benefits of using CDIM for biomarker discovery, one main disadvantage using the medium was observed. The CDIM was not chemically stable at 37°C for several days and in addition, the CDIM became more complex during storage. Unsupervised multivariate data analysis of replicates incubated under similar conditions as the fermentations showed a large within batch variability. As indicated in figure 7.5, the larger the degree of fermentation, the closer the biological replicates clustered. The CDIM is a rich medium containing high concentrations of glucose as well as the full range of essential amino acids, nucleotides and vitamins to cover the comprehensive nutrient demand of lactic acid bacteria. Compounds like the vitamins and cysteine are labile in solution. Furthermore, the reducing sugar, glucose, will react with amino acids, hereby altering the composition over time. This underlines indeed the importance of including un-inoculated sample blanks in this type of studies.

### 7.4 The antifungal properties of the LAB A and PAB A co-culture

In summary, the antifungal effect of co-culture of LAB A and PAB appears to be composed of several factors. Diacetyl, either directly secreted or formed from secreted alpha-acetolactate seems to provide the major antifungal effect independent of pH (neutral or acidic). The acidification of the medium (by production of lactic acid) in combination with production of 2-hydroxy acids appears to contribute weakly to the overall effect, as seen by a decrease in the color development
of the mold spores. Consumption and potentially full depletion of specific nutrients also seems to retard mold development. Whether the factors observed are synergistic or additive has not been assessed.

Besides diacetyl, no highly potent single component was identified to provide a major contribution to the antifungal effect of LAB A.

Based on microbiological observations, the presence of mold hyphae did not enhance any additional antifungal effect. The bacteria generated the antifungal effect based on the properties (genome) of the LAB strain, and the co-fermentation with the PAB resulted in an enhanced metabolism which resulted in increased secretion of antifungal factors. Other aspects of the interspecies interaction between PAB A and LAB A have not been investigated, e.g. no studies have been made on the potential changes in metabolism in PAB A caused by presence of LAB A ferments. The bacterial production of alpha-acetolactate and hence diacetyl as well as 2-hydroxy acids occurred towards the end of the exponential phase, typically at pH below 5 indicating that the antifungal effect could be part of the stress response to acidic conditions.

As previously reported (and well documented internally), the presence of bacterial cells was required to obtain the antifungal effect. No experiments were performed with addition of dead cells. With live cells in the medium, the concentration of diacetyl decreased over time. The loss of diacetyl could be caused by evaporation and/or irreversible reactions with components in the exometabolome, e.g. residual arginine.

Apparently, the bacterial cells lose the ability to compensate for the loss of diacetyl over time. The diacetyl levels converged towards a constant diacetyl concentration of ca. 10 pg/g, which corresponded to the residual concentration after removal of cells.

All observations listed above were drawn from studies of the co-culture in the chemically defined interaction medium, CDIM. Only a few studies were performed with measurements and addition of diacetyl in an actual food system, yoghurt. The studies were in accordance with the observations made in CDIM, but not sufficient to conclude that the antifungal effect is composed as in the CDIM.

EXAMPLE 8 - CONCLUSION
The inventors’ aim was to develop and apply a chromatography mass spectrometry based metabolomic footprint workflow for the investigation of the mechanisms behind the antifungal properties of a co-culture, consisting of *Lactobacillus paracasei* (LAB A) and *Propionibacterium freudenreichii* subsp. *shermanii* (PAB A).

The strategy was to correlate biological responses to chemical profiles of the exometabolome of the co-culture. In actual food applications, the antifungal effect was observed in a complex system consisting of a complex and inhomogeneous matrix (fermented milk), starter culture (two or more LABs), the co-culture with antifungal effect (LAB A and PAB A) and the fungi. In order to reduce the number of variables in the system and to allow chemical and biological measurements with a minimum of sample preparation, a biological model system was developed based on a chemically defined interaction medium (Example 1F). The growth of mold on the surface of bacterial ferments served as the biological response, which was documented by use of multispectral imaging. Quantification of mold growth was based on a developed spectral clustering algorithm (Example 1J).

Metabolic footprinting was performed on a combination of instrumental platforms and methods. The instrumentation and methods were selected based on expected compound classes in the exometabolome with LC/MS as the primary technique. Hydrophilic interaction and reversed phase UPLC in combination with high resolution MS via electrospray positive and negative were chosen as the four analytical modes (Honore ei a/ 2013 Anal Bioanal Chem. 2013 Oct;405(25):8151-70). Shortcomings in the coverage of metabolites, especially in terms of low molecular weight and volatile compounds were covered by supplementary analyses with pre-derivatisation (ethyl esters) and headspace sampling GC/MS. Quality assurance for the non-targeted data acquisition was based on use of randomization of samples and use of pooled quality control standards. In spite of several optimizations, the HILIC method suffered from insufficient sequence stability and several datasets failed QC requirements. Data from untargeted LC/MS analyses was processed using a strategy of feature selection and subsequent multivariate data analysis. Two software tools for feature selection were compared on similar dataset. MZmine2 outperformed the other due to higher precision in the integration and GAP filling functions. Exploratory multivariate data analysis was primarily done by PCA and search for biomarkers or antifungal properties were done using PLSR. The full workflow allowed for grouping of LAB phenotypes (unpublished) and genotypes (Example 1H).
The process for investigation of the antifungal properties was an iterative and combined effort of analytical chemistry and microbiology. Screening experiments by microbiology formed the basis for larger scale experiments where sampling, documentation and chemical analysis provided data for interpretations and hereby new hypotheses to test in experiments.

The antifungal properties of the co-culture in CDIM were composed of several factors. LAB A in the co-culture produced the single most important factor under the tested conditions, which was diacetyl, either directly secreted or formed from secreted alpha-acetolactate by oxidative decarboxylation. Diacetyl in concentrations down to ca. 45 µg/g provided a significant delay in mold outgrowth and development in acidic and neutral media (Example 1G). Furthermore, the acidification of the medium by production of lactic acid and production of other 2-hydroxy acids contributed weakly to the overall antifungal effect.

The production of diacetyl and 2-hydroxy acids occurred without any perturbation of the microbiological ecology and the rate of production increased towards the end of the exponential phase at pH below 5.

Fermentation of LAB A in the presence of PAB A pre-fermented ferment enhanced the growth rate of LAB A and, hereby, the increased production of diacetyl and 2-hydroxy acids. Based on microbiological observations, the presence of mold hyphae did not enhance any additional antifungal effect. The presence of LAB A cells was needed to obtain a concentration of diacetyl high enough to inhibit mold. With the presence of cells, diacetyl concentration peaked at ca. 70 µg/g within the first two days and then declined during 5-6 days time to a concentration of ca. 10 µg/g whereby the inhibitory effect was lost.

All the above-mentioned observations were made in CDIM. Initial work was started to validate the observations in yoghurt. Inhibition of mold in a food system (yoghurt and acidified milk) was obtained by spiking with diacetyl. The effect corresponded to one observed with yoghurt co-inoculated with the LAB A strain. Relative quantification demonstrated an increase in diacetyl production in yoghurt co-inoculated with LAB A compared to yoghurt without.

In order to search for antifungal properties beyond diacetyl, cell-free ferments of LAB A together with two other strains of *Lactobacillus paracasei* were subjected to metabolic footprinting and mold growth assay. Removal of the bacterial cells significantly decreased the inhibition, and variation in
effect between the strains could only be observed as differences in the number of matured spores after five days of mold growth.

The three LAB strains could be grouped according to their relative consumption of nutrients and production of metabolites, mainly six 2-hydroxy acids. The variations in mold growth correlated to the grouping of the three LAB strains. All of the six 2-hydroxy acids possessed antifungal activity. Two of the 2-hydroxy acids, (2-hydroxy-3-phenyl)propanoic acid and 2-hydroxy-3-(4-hydroxyphenyl)propanoic acid, were previously identified as antifungal in a co-culture similar to the tested co-culture (as well as in other LABs with antifungal effect). A third, 2-hydroxy-4-methylpentanoic acid was previously identified as antifungal in a \textit{Lb. plantarum} strain. The remaining three 2-hydroxy acids were not previously described as antifungal, namely 2-hydroxy-3-methylbutanoic acid, 2-hydroxy-3-(1H-indol-3-yl)propanoic acid and 2-hydroxy-(4-methylthio)butanoic acid. The concentrations of the acids were below 65 mg/L and in too low concentrations to individually provide the antifungal effect (Example 1H).

The study enabled the identification of one major and three minor, non-previous assigned contributors to the antifungal effect of \textit{Lactobacillus paracasei}, and hence of the co-culture with antifungal properties.

All in all, the ability to differentiate both LAB phenotypes and related LAB genotypes by metabolic footprinting was demonstrated. The strategy to apply metabolomic footprinting on a model system with minimal sample preparation and correlation to biological response proved to be a valuable supplement to the otherwise applied strategy of bioassay guided fractionation.

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in biochemistry and molecular biology or related fields are intended to be within the scope of the following claims.
DEPOSITS

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL Pigious

Dentsco  Deutschland GmbH
DuPont Nutrition and Health
ausch-Joiiannsen-Str.  25899 Niort
Germany

I. IDENTIFICATION OF THE MICROORGANISM

Identity citation reference given by the DEPOSITOR:
DGCCC95

Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
DSg 28601

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

- ( ) a scientific description
- ( ) a proposed taxonomic designation

(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depository Authority accepts the microorganism identified under I above, which was received by it on 2014-01-25 (Date of the initial deposit)*

IV. REQUEST FOR CONVERSION

The microorganism identified under I above was received by the International Depository Authority on

( ) + day(s) prior to this deposit. The original deposit of the deposit was received by it on 2014-03-28 (date of original deposit)

( ) + day(s) after this deposit. The original deposit of the deposit was received by it on 2014-03-28 (date of receipt of request)

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures
Address: Inhoffenstr. 7 B
D-38124 Braunschweig

Signature(s) of person(s) having the power to represent the International Depository Authority or of author and official(s):

Date: 2014-03-28

* Where Rule 5.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

Form PCT-EP (sole page) 02/2012
### INTERNATIONAL FORM

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<th>II. IDENTIFICATION OF THE MICROORGANISM</th>
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<tr>
<td>Name: Danisco Deutchland GmbH</td>
<td>Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 28601</td>
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<tr>
<td>Address: DuPont Nutrition and Health Busch-Johannsen-Str. 1 25889 Niebüll Germany</td>
<td>Date of the deposit or the transfer*: 2014-03-25</td>
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</table>

### VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on 2014-03-25.

\[
\begin{align*}
(x) & \quad \text{viable} \\
(\_\_\_\_) & \quad \text{no longer viable}
\end{align*}
\]

### IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED*

### V. INTERNATIONAL DEPOSITARY AUTHORITY

| Name: Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures | Signatory(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): |
| Address: Infostation 7 B D-38124 Braunschweig | |

* Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

* In cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.

** Mark with a cross the applicable box.

* Fill in if the information has been requested and if the results of the test were negative.

Form DSMZ-IIpa doc (p. 3) 02/2012
OATH AND DECLARATION

We, Dr. deem Plock, Managing Director, and Michael Paulsen, Prokurist, authorised signatories of Danisco Deutschland GmbH of iusGh-Johannsen-Stesse 1, D-25899 Niebüll, Germany [hereafter named Danism Deutschland GmbH] hereby make the following oath and declaration:

We confirm and declare that Danisco Deutschland GmbH has deposited and is the lawful owner of the following biological material(s):

<table>
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<th>Deposited material</th>
<th>Accession Number</th>
<th>Deposit Date</th>
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</thead>
<tbody>
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<td>Lactobacillus paracasei ssp. paracasei</td>
<td>DSM 25601</td>
<td>25 March 2014</td>
</tr>
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We further confirm and declare that Danisco Deutschland GmbH gives DuPont Nutrition Biosciences ApS of Langebroade 1, DK-1411 Copenhagen K, Denmark [hereafter named DuPont Nutrition Biosciences ApS] unconditional and irrevocable right and consent to use and make available to the public the above deposited biological material(s) for the extent necessary for, in the name of DuPont Nutrition Biosciences ApS, prosecuting application(s) for and obtaining patent(s) related to the invention with the internal reference No. NB40641-PRJ or any application(s) or patent(s) derived or claiming priority there from.

SWORN and SIGNED in the presence of a witness.

Signature: [Signature]
Date: 31.03.2013
Place: Niebüll

Signature: [Signature]
Date: 01.04.14
Place: Niebüll

Witness Name and Address
1. [Name and Address]
Witness Signature and date
2. [Signature]
Date: 31.03.2014

Witness Signature and date
3. [Signature]
Date: 01.04.14
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CLAIMS

1. A bacterium of species *Lactobacillus paracasei* strain DGCC 695 deposited as DSM 28601 at Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures or a functional equivalent thereof.

2. A combination of bacteria comprising the bacterium as defined in claim 1 and a further bacterium.

3. The combination according to claim 2, wherein the further bacterium is selected from a lactic acid bacterium, a *Propionibacterium*, a *Bifidobacterium* or a mixture thereof.

4. An inoculum comprising a bacterium according to claim 1 or a combination according to claim 2 or 3.

5. A culture medium comprising a bacterium according to claim 1 or a combination according to claim 2 or 3.

6. A food product or a feed product comprising a bacterium according to claim 1 or a combination according to claim 2 or 3.

7. A method of producing a fermented milk-based product, comprising contacting a bacterium as defined in claim 1, or a combination as defined in claim 2 or claim 3, or an inoculum according to claim 4 or a culture medium according to claim 5, with milk or a component of milk.

8. A fermented milk-based product obtained or obtainable by the method according to claim 7.

9. A method of producing a fermented food product or feed product, comprising contacting a bacterium as defined in claim 1, or a combination as defined in claim 2 or claim 3, or an inoculum according to claim 4 or a culture medium according to claim 5, with a substrate for the food product or the feed product.

10. A fermented food product or feed product obtained or obtainable by the method according to claim 9.
11. Use of a bacterium as defined in claim 1, or a combination as defined in claim 2 or claim 3, or an inoculum according to claim 4 or a culture medium according to claim 5, to control the growth of one or more contaminating microorganisms (such as a bacteria, yeast or mould) in a food product or a feed product.

12. Use of a bacterium according to claim 1, or a combination according to claim 2 or 3, or an inoculum according to claim 4 or a culture medium according to claim 5, for preserving a food product or a feed product.
Figure 1.1a

2132

Figure 1.2

2132

Figure 1.3
Figure 1.9

**pH and absorbance after 20 h fermentation at 37 °C**

![Graph showing pH and absorbance](image)

Figure 1.10

![Image showing cell growth](image)

+ Cells

Cell free
Figure 1B.1

Figure 1B.2

Figure 1B.3
Figure 1B.7

Figure 1B.8

Figure 1B.9
Figure 1B.10

Figure 1B.11

Figure 1B.12
Figure 1B.13

Figure 1B.14
Figure 1D.1

Figure 1D.2
Analytical platform A

Analytical platform B

Qualitative
Quantitative

Fig 1E.1

Vis
NIR

covarygram

correlation/covariation

Fig 1E.2
Fig 1E.3
Fig 1E.6
Fig. 1F.4

1.43±0.10  1.78±0.12
0          0.77±0.03

Fig. 1F.5

1.38±0.04  0  0.98±0.07  0.71±0.08

Fig 1F.6
Fig 1G.1

Fig 1G.2
Fig 1H.2

Samples/Scores Plot of RPCombS

ESI Pos

Scores on PC 1 (55.99%) vs Scores on PC 2 (27.46%)

- Higher biomass, lower pH and less mold

ESI Neg

Scores on PC 1 (55.60%) vs Scores on PC 2 (27.28%)

- Higher biomass, lower pH and less mold

Variables/Loadings Plot for RPCombS

ESI Pos

- Aromatc amino acids

ESI Neg

- Branched chain 2-hydroxy acids

- Nutrients and intermediates

- Branched chain amino acids

- Aromatic 2-hydroxy acids metabolites
Figure 3.1

Figure 3.2
Figure 3.3

Figure 3.4
Figure 5.1

Figure 5.2
Figure 5.3
RPC ESI pos

RPC ESI neg

HILIC ESI pos

HILIC ESI neg

Figure 5.4
Equilibration of system  
Bracket 1 with randomised test and QC samples within  
Bracket 2  

Figure 5.5  

Data acquisition  
Raw data export  
Filtering  
Feature Detection  
Peak alignment  
Normalization  

Pre-processing  

Conversion of instrument vendor file format to generic format e.g. cdf, mzXML, mzData  
Deconvolution, dimension reduction and alignment in terms of retention time and m/z ratio  
Correction for unintended biological and analytical variation  
Scaling to compensate for differences in fold changes for different metabolites  
Selection and evaluation of algorithm results, e.g. PCA, PLS-DA and PLS-DVA to explore and visualize data  
Variable selection and assignment of chemical identity to biomarkers  

Mining  

Biological Interpretation  
Hypothesis generation  

Figure 6.1
Figure 6.2

Figure 6.3
Figure 6.4

Figure 6.5
Figure 6.6

Figure 6.7
Figure 6.8
Fig. 6.9 continued
Figure 6.10
Figure 6.11

Figure 6.12
Figure 6.13
Figure 7.1

Figure 7.2
Figure 7.3

**Penicillium solitum**

DCS 302

Yoghurt

Milk acidified to pH 4.5

---

**Penicillium sp.**

DCS 1541

Yoghurt

Milk acidified to pH 4.5

Figure 7.4
Figure 7.5

Figure 7.6
Figure 7.7
**INTERNATIONAL SEARCH REPORT**

**International application No**

PCT/EP2015/061762

**A. CLASSIFICATION OF SUBJECT MATTER**

INV. C12N1/20 A23C9/123 A23L3/3571 A01N63/00 A61K35/747

**ADD.**

According to International Patent Classification (IPC) and/or both national classification and IPC

**B. FIELDS SEARCHED**

**Minimum documentation searched (classification system followed by classification symbols)**

C12N A23C A23L A01N A61K C12R

**Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched**

**Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)**

EPO-Internal, BIOSIS, Sequence Search, EMBASE, FSTA, WPI Data

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

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Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents:
  
  **"A"** document defining the general state of the art which is not considered to be of particular relevance
  
  **"E"** earlier application or patent but published on or after the international filing date
  
  **"L"** document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
  
  **"O"** document referring to an oral disclosure, use, exhibition or other means
  
  **"P"** document published prior to the international filing date but later than the priority date claimed

Date of the actual completion of the international search 13 October 2015

Date of mailing of the international search report 28/10/2015

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL-2280 HV Rijswijk
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Fax: (+31-70) 340-3016

Authorized officer

Madruga, Jaime
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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT
### DOCUMENTS CONSIDERED TO BE RELEVANT

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**INTERNATIONAL SEARCH REPORT**

**Box No. I**  
**Nucleotide and/or amino acid sequence(s)** (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
   a. [X] forming part of the international application as filed:
      - [✓] in the form of an Annex C/ST.25 text file.
      - [ ] on paper or in the form of an image file.
   b. [ ] furnished together with the international application under PCT Rule 13fer1 (a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
   c. [ ] furnished subsequent to the international filing date for the purposes of international search only:
      - [ ] in the form of an Annex C/ST.25 text file (Rule 13fer1 (a)).
      - [ ] on paper or in the form of an image file (Rule 13fer1 (b) and Administrative Instructions, Section 7.13).

2. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments: