Near infrared and mid-infrared spectroscopy in the study of cholesterol removal ability of some lactic acid bacteria strains

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Introduction

The consumption of food rich in fat, especially saturated fatty acids and cholesterol, is one of the most important risk factors for coronary heart disease (CHD). The cholesterol molecule has an important role in maintaining the integrity of cell membranes that regulate permeability and fluidity, and is involved in many metabolic pathways such as precursor of steroids, bile acids and vitamin D. Consumers are becoming more concerned about the excessive intake of fat in the diet and demand food with a lower fat content and with functional properties which can have beneficial effects.[1] The consumption of dairy products containing probiotic bacteria has been proposed as a means to lower cholesterol.[2] Probiotics are defined as a "live microbial supplement that beneficially affects the host by improving its intestinal microbial balance". Many authors have shown how these bacteria are able to decrease blood cholesterol content, proposing several in vitro mechanisms of cholesterol-lowering by probiotic bacteria, even if the exact mechanism remains unclear.[2-4] Recent papers have studied the capacity for strains isolated from dairy matrices to remove cholesterol during growth. In particular, Belviso *et al*.[1] focused on *Lactobacillus plantarum*. *Lactobacillus plantarum* is a member of the facultative heterofermentative group of lactobacilli and it is present in a variety of environmental niches, including dairy, meat, fish, and many vegetable or plant fermentations. Moreover, strains of *L. plantarum* are tolerant of gastric transit and colonise the intestinal tract of humans and other mammals.[5]

Methods for investigating whether cholesterol was assimilated by bacterial strains, or if it remained free in the culture medium, have been proposed. Many authors used the method of Rudel and Morris (1973);[6] cholesterol can be estimated by visible spectroscopy using *o*-phthalaldehyde reagent after extraction of unsaponified matter (cholesterol) with an organic solvent such as hexane. The extraction method is time consuming, and hence rapid spectroscopic methods have been proposed using mid-infrared (MIR) and near infrared (NIR) techniques.[7-9] The aim of this study was to assess, by the use of NIR and MIR spectroscopic techniques, whether strains with different abilities to remove cholesterol could show changes in the cell wall and if it was possible to determine the amount of the cholesterol removed.

Materials and Methods

Samples

Four *L. plantarum* strains (Lp752, Lp885, Lp995, Lp997) belonging to the collection of CRA-FLC (Lodi, Italy) and isolated from Italian dairy products, and the type strain ATCC14917, stored as frozen stock at –80°C, were cultivated overnight in MRS broth (Merck, Darmstadt, Germany) at 30 ± 1°C. After growth, 2% (v/v) of fresh culture was transferred in either MRS or MRS broth supplemented with a stock solution of cholesterol (10% w/v) to obtain a final concentration of 0.1 $g.l^{-1}$. The cholesterol stock solution (1 $g.l^{-1}$) was prepared by dissolving 100 mg of cholesterol (Sigma-Aldrich, Milan, Italy) in an aqueous solution of 26.4% ethanol and 12% Tween 80 (polyoxyethylene sorbitan monooleate) (Sigma-Aldrich, Milan, Italy). Two groups of samples were analysed after growth for 24 h at 30 ± 1°C: A) cells harvested by centrifugation (7000 rpm for 10 min at 4°C) and washed twice with ultrapure water; and B) culture supernatant. The pH values of samples from group B averaged 3.9 ± 0.2.

MIR spectroscopy

One gram of sample A was distributed on ZnSe tablets (∅ 13 mm, h 2 mm; PIKE Technologies, Maddison, WI, USA) for collecting attenuated total reflectance Fourier-transform infrared (ATR-FTIR) spectra. Each spectrum was an accumulation of 128 scans between 900 and 1750 cm^{-1} at a spectral resolution of 4 cm^{-1}, and was collected at room temperature using a FT-IR 420 spectrometer (JASCO Europe, Cremella, Como, Italy).

Reference paper as:
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NIR spectroscopy

FT-NIR spectra were collected on samples from group B in transmission mode (pathlength 0.2 mm, 32 scans, resolution 8 cm^{-1}, 6 replicates) with a NIRFlex N-500 spectrometer (BUCHI, Italy). Samples were measured between 4000 and 10000 cm^{-1} while under a controlled temperature (40 ± 1°C). Spectra were collected with NIRWare Operator 1.2 (BUCHI, Italy) and processed with The Unscrambler 9.2 (Camo Inc., Oslo, Norway).

Data processing

The average spectrum of water or supernatant was subtracted from the MIR spectrum of sample A (average of 5 replicates), depending on the matrix, and the adjusted sample A spectra were then smoothed (Savitzky-Golay, 7 points, polynomial order 0). NIR data were processed in order to evaluate qualitative differences between culture supernatants (in absence and in presence of cholesterol). NIR spectral data were transformed by standard normal variate (SNV), smoothed (Savitzky-Golay second derivative, 8 points, polynomial order 2), mean centered, and then analysed with principal component analysis (PCA).

Results and Discussion

Lactic acid bacteria (LAB) are Gram+ microorganisms and their cell walls are primarily made of peptidoglycan (ca. 40–80% of the dry weight of the wall), which is a polymer of *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM). Other important constituents are: teichoic acid, a polymer of glycopyranosyl glycerol phosphate, and teichuronic acid, which is similar to teichoic acid but replaces the phosphate functional groups with carboxyls, and surface proteins.[6] Therefore, the principal functional groups found in MIR spectra were: carboxyl, hydroxyl, phosphate and amide. In particular, phosphate group in bacteria can exist both in inorganic forms such as orthophosphate and its oligomers and in organic forms such as phosphate mono- and diesters. The principal absorption bands, identified in the second derivative of MIR spectra, are shown in Table 1.[10-11]

Table 1. Absorption bands of functional groups recognisable in the spectra of *Lactobacillus plantarum* before and after the addition of cholesterol. References in parentheses.

Absorption bands (cm^{-1})	Vibrational modes
950–1150	Asymmetric and symmetric stretching of PO_2^- [10]
1153	C-O stretching in C-OH group[13]
900–1200	C-O-C and C-O of various polysaccharides[12]
1215	Interaction of O-H bending and C-O stretching in C-O-H group,[8] C-OH stretching vibration of carboxylic acid groups,[11] stretching of P=O bond in phosphate[10]
1220–1330	Amide III band components of proteins:[1] 1220-1250cm^{-1} β sheets, 1250-1270 cm^{-1} random coils, 1270–1295 cm^{-1} β turns,1295–1330 cm^{-1} α helix[14]
1240	PO_2^- stretching of phosphodiesters,[12] C–O stretching in carboxylic acid[13]
1372	Interaction of O-H bending and C-O stretching in C-O-H group, [8] CH_3 symmetric bending[13]
1396	Symmetric stretching of COO^- [10]
1407	=C–H in-plane bending[13]
1418	C-O-H in-plane bending, carboxylic acids[13]
1454	CH_2 scissoring[10]
1566	C=C stretching in alkenes[13]
1548	Amide II band[12]
1637	Amide I of β-pleated sheet structure[12]
1743	C=O stretching of esters[12]

Preliminary measurements were performed on five strains of *L. plantarum* (Lp752, Lp885 Lp995, Lp997 and ATCC14917), which were cultivated either with or without cholesterol. MIR spectra measured from centrifuged pellets (matrix spectrally subtracted) showed an interesting change between 1180 and 1300 cm^{-1}, whose entity depended on the strain characteristics. In particular, a shift in band position from 1240 cm^{-1} towards 1217 cm^{-1} was observed, and attributed to the formation of hydrogen bonds between phosphate and hydroxyl groups in cholesterol. The formation of hydrogen bonds weakens all the bonds involved and shifts absorption bands towards lower energy.

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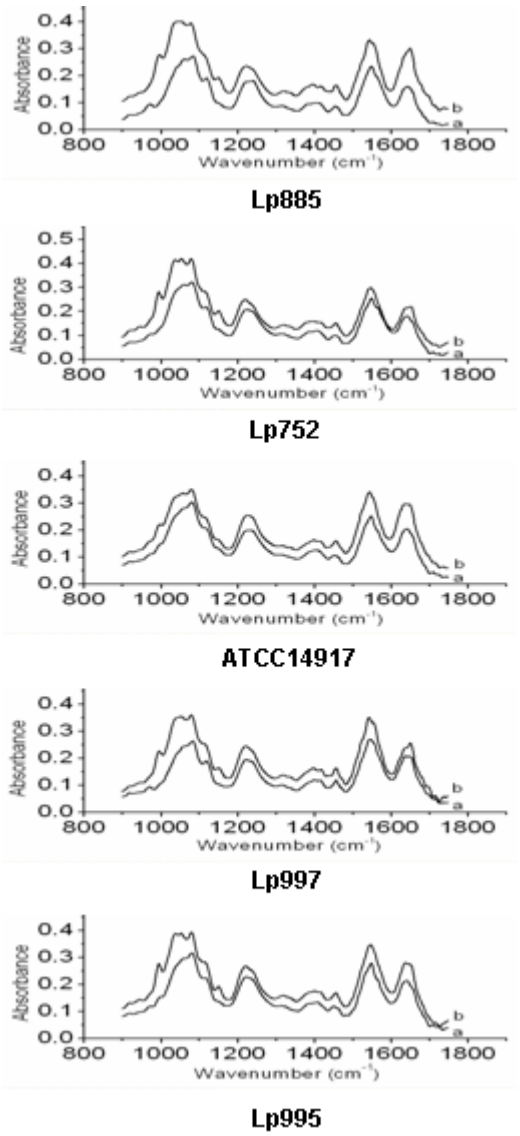

Figure 1. MIR spectra of Lp995, Lp997, ATCC14917, Lp752 and Lp885 strains cultivated without (a) and with (b) cholesterol (Note: absorbance scale has been shifted to facilitate comparison).

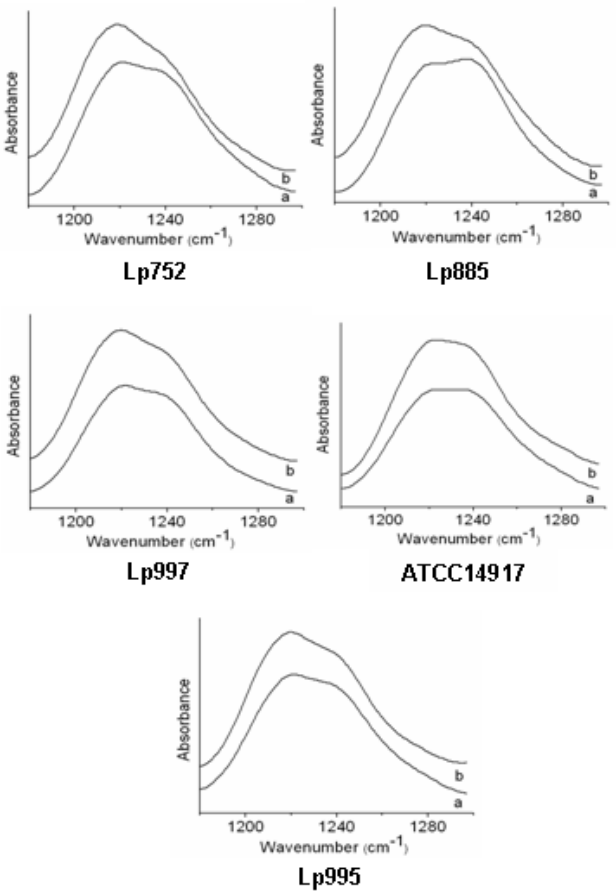

Figure 2. Detail of MIR spectra between 1180 and 1300 cm^{-1} for Lp995, Lp997, ATCC14917, Lp752 and Lp885 strains cultivated without (a) and with (b) cholesterol (Note: absorbance scale has been shifted to facilitate comparison).

Hydrogen bond redshifts had a minimal effect on MIR spectra of Lp995, Lp997 and ATCC14917, but were prominent in spectra of Lp752 and Lp885 (Figures 1 and 2). Spectral observations agreed with expectations for cholesterol removal, demonstrating the increased capacity of Lp752 and Lp885 to remove cholesterol.[15] We noted that initial band shapes differed among strains, which likely relates to different cell wall compositions and potentially explains different capacities for removing cholesterol from the culture broth. Other spectral variations between strains were evident between 900 and 1150 cm^{-1}, and measurements performed on washed cells of Lp885 suggested that some variations were due to an interference of the matrix (variations were removed after washing; data not shown). The most important range in a MIR spectrum for tracking cholesterol interaction was 1180 to 1300 cm^{-1}.

The supernatants obtained after centrifugation of three strains (Lp885, Lp995, ATCC14917) cultivated with and without cholesterol were analysed with NIR spectroscopy. Preliminary data, processed by PCA, showed the possibility to separate the supernatants obtained both in absence (supernatant a) (Figure 3) and in presence (supernatant b) of cholesterol (Figure 4), highlighting differences in metabolism and in the capacity to remove cholesterol among strains. The difference between strains in Figures 3 and 4 was observed along principal component one (PC1; 97% of total variance explained) for ATCC14917 and for the samples sets Lp885 and Lp995; separation for Lp885 and Lp995 was essentially obtained along PC2 (3% of total variance explained).

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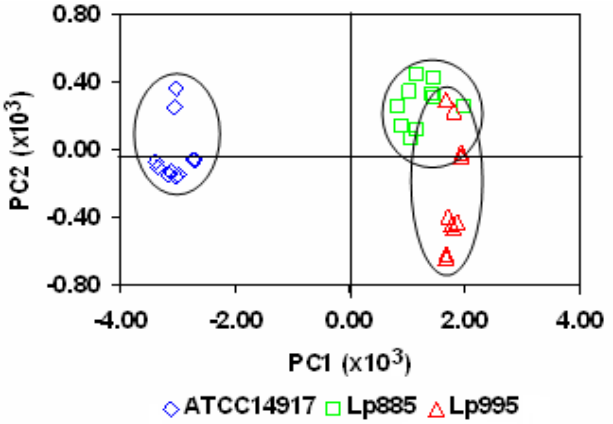

Figure 3. Scores plot obtained from a PCA performed on 30 samples (10 of ATCC14917, 10 of Lp885 and 10 of Lp995) of supernatant a.

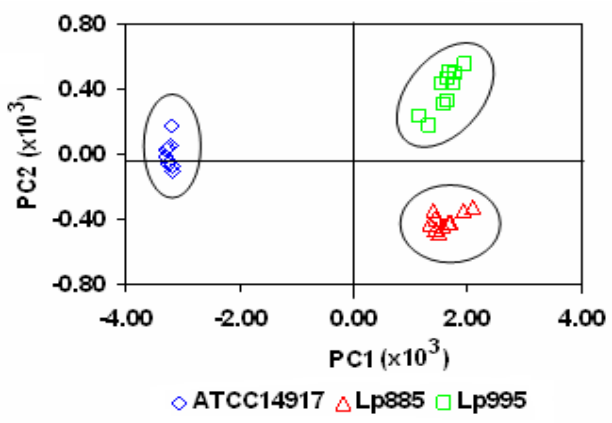

Figure 4. Scores plot obtained from a PCA performed on 30 samples (10 of ATCC14917, 10 of Lp885 and 10 of Lp995) of supernatant b.

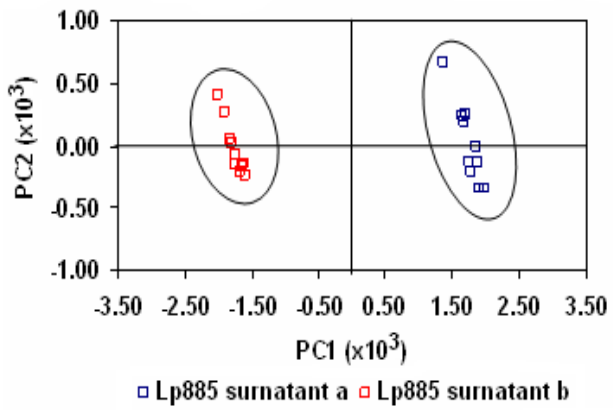

Figure 5. Scores plot obtained from a PCA performed on 20 samples of Lp885 supernatant (10 of supernatant a and 10 of supernatant b).

The score plot obtained by the PCA analysis performed on 20 samples of Lp885 supernatant without (a) and with (b) of cholesterol is shown in Figure 5. The two supernatants were separated along PC1, which was able to explain 97% of the total variance. A more detailed study is in progress to verify the feasibility of NIR spectroscopy for a rapid determination of cholesterol remaining in supernatants.

Conclusions

Preliminary results support the utility of NIR and MIR spectroscopy for studying the removal of cholesterol by some strains of LAB, and suggest that the removal mechanism is an interaction between cholesterol and cell walls involving hydrogen bonds. Further studies will be addressed to confirm these observations and to develop a fast method, based on NIR spectroscopy, for the determination of cholesterol.

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