Importance of raw milk lactic acid bacteria for cheesemaking: a new approach for their detection

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Parmigiano Reggiano cheese

Parmigiano Reggiano is a hard cheese, obtained from cooked raw cow's milk, partially skimmed milk.

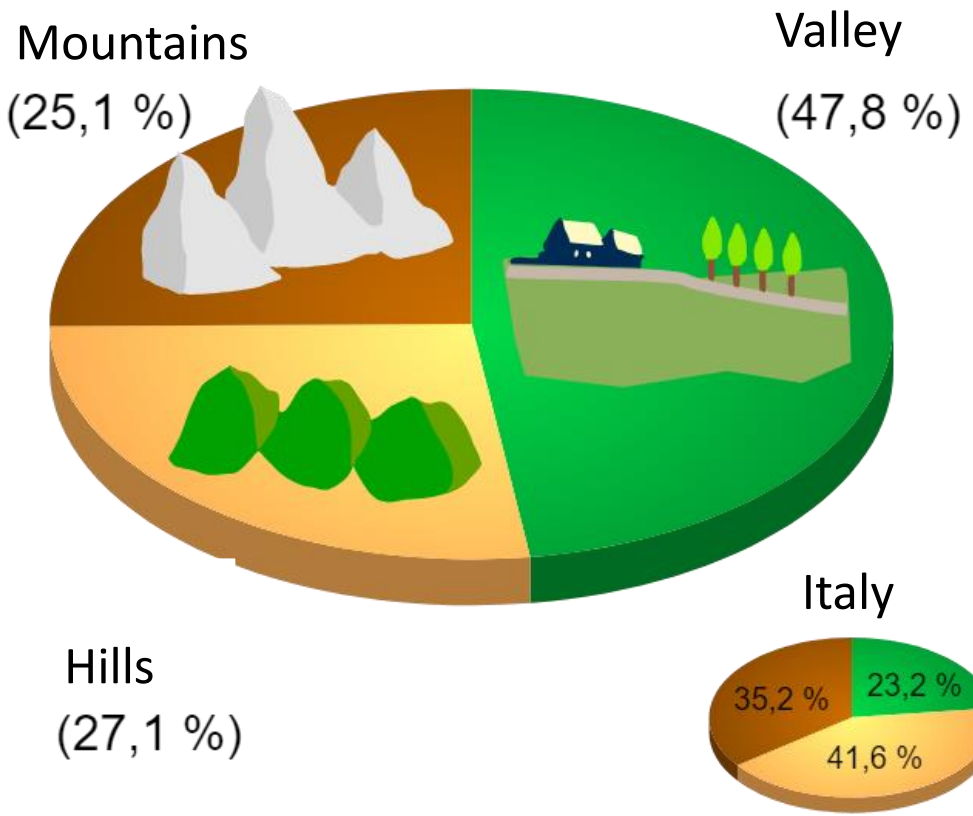
The production of milk, take place exclusively in the provinces of Parma, Reggio Emilia, Modena, Bologna to the left of the river Reno and Mantua to the right of the river Po.

Only raw milk produced in the area of origin is used to produce Parmigiano Reggiano cheese.

This area hosting 3000 farms where the cattle are fed on locally grown forage.

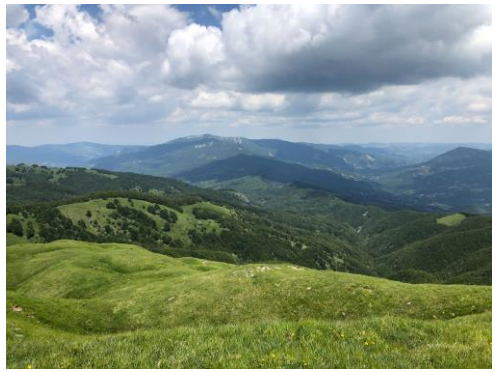
The feeding of cattle complies with the norms of a strict specification that bans the use of silage and fermented feeds

The land of production: Emilia-Romagna



The production of Parmigiano Reggiano takes place between mountains, valley and hills, this diverse environments is strongly link to the unique characteristics of this product.

Reservoir of biodiversity that makes Parmigiano Reggiano unique



Link to the land of production: PDO-cheese

To bear the designation “Parmigiano Reggiano PDO,” the cheese has to be made respecting strict rules.

- First of all, it has to be produced in the area of origin

Class 1.3. Cheeses

3.2. *Description of the product to which the name in (1) applies*

‘Parmigiano Reggiano’ is a hard cheese made from raw cow’s milk, which is partially skimmed by allowing the cream to rise to the surface. The cheese is scalded and slowly matured. The milk must not undergo any heat treatment and has to come from cows fed primarily on fodder obtained in the area of origin. The cheese must be matured for at least 12 months. ‘Parmigiano Reggiano’ can be sold whole, in portions or grated.

‘Parmigiano Reggiano’ has the following characteristics:

— The cheeses are cylindrical with a slightly convex or virtually straight heel and flat faces with a slightly raised edge.

— The flat faces have a diameter of 35 cm to 45 cm and the heel height is 20 cm to 26 cm.

— The minimum weight of each cheese is 30 kg.

— The outer rind is a natural straw colour.

— The rind is approximately 6 mm thick.

— The body of the cheese is between light straw-coloured and straw-coloured.

— The cheese’s characteristic aroma and taste are fragrant, delicate and flavoursome without being pungent.

— The body of the cheese is fine-grained and flaky in texture.

— The minimum fat content in dry matter is 32 %.

— The cheese contains no additives.

— It has a cyclopropane fatty acid ratio of less than 22 mg per 100 g of fat (already accounting for uncertainty of measurement, gas chromatography-mass spectrometry (CG-MS) method).

3.3. *Feed (for products of animal origin only) and raw materials (for processed products only)*

The cows are to be fed primarily on fodder from the defined geographical area, specified by quantity and quality.

At least 75 % of fodder dry matter must be produced within the geographical area.

Feedstuffs may make up at most 50 % of the dry matter of the feed ration.

The use of silage of any kind is banned.

Cow’s milk, salt, calf rennet.

The milk is from cows reared in the defined geographical area.

3.4. *Specific steps in production that must take place in the identified geographical area*

The farms that rear the dairy cows whose milk is to be processed into ‘Parmigiano Reggiano’ must be located within the defined geographical area.

The milk must be produced and processed within the defined geographical area. The milk from the evening and morning milkings is delivered to the dairy raw and unskimmed, observing the production specifications. The milk from the morning milking is placed in copper vats and mixed with the milk from the previous evening, partially skimmed by allowing the cream to rise to the surface. The fat-to-casein ratio of the milk in the heating vats, calculated as the weighted average of all batches in a given day’s production, cannot exceed 1,1 + 12 %. The whey from the previous day’s cheesemaking is added to the milk; the use of starter cultures is not permitted. Following coagulation, for which only calf rennet can be used, the curds are broken up and scalded. Once the cheese mass has sunk to bottom of the vat, it is set in moulds. Markings are then stamped onto the cheeses. After some days, the cheeses are soaked in brine and then matured for at least 12 months.

The minimum 12-month maturing period must take place in the defined geographical area.

After the minimum maturing period, a quality inspection is performed to check compliance with the production specifications.

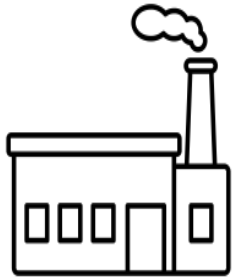
3.5. *Specific rules concerning slicing, grating, packaging, etc. of the product the registered name refers to*

‘Parmigiano Reggiano’ can be sold whole, in portions or grated.

In the interests of consumer protection, in order to guarantee the authenticity of pre-packaged, grated or portioned ‘Parmigiano Reggiano’ placed on the market, grating, portioning and subsequent packaging must take place in the defined geographical area. This is necessary because the markings that identify a whole cheese as ‘Parmigiano Reggiano’ are lost or are not visible on grated cheese or portions, so the origin of the pre-packaged product has to

How the consortium works

2.356 Farmers



309 cheese factory

4.002.270 produced cheeses/year

The consortium members are for their own choice part of the consortium that includes farmers, cheese makers, and ripeners who are in the area of production

Main functions:

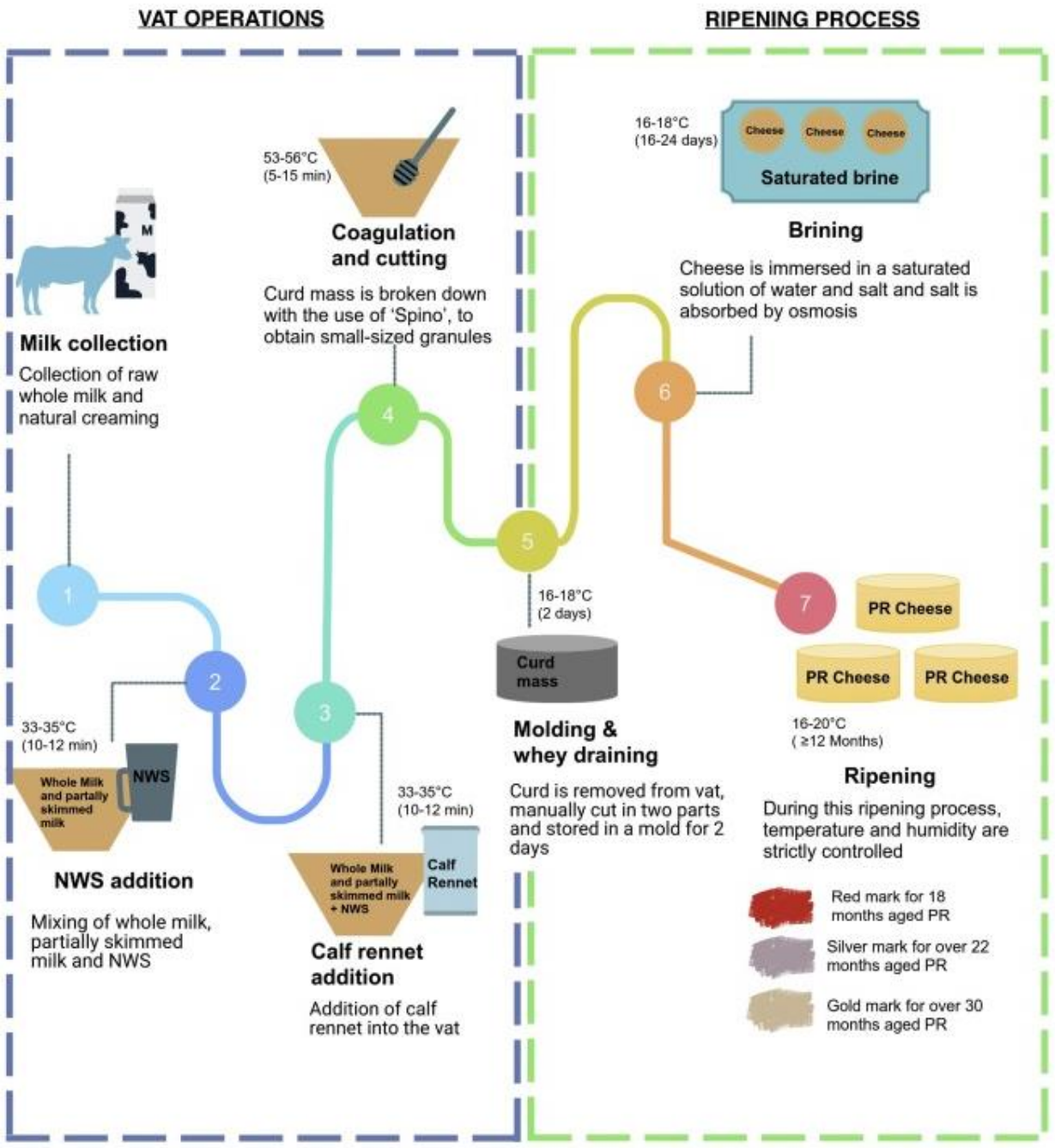
- Protection of the PDO label
- Supervision on the farmers and cheesmaker to be sure that they follow the PDO regulation
- Valorization of the production
- …..

1. Milk is collected twice a day (temperature min 18°C), only the milk from the evening milking is partly skimmed by spontaneous flotation of milk fat globules

2.Up to 10% of the whole milk from the morning is mixed with that of the evening.
It takes about 550 litres of milk to produce each wheel of Parmigiano Reggiano

3.The milk slowly and naturally coagulates with the addition of rennet and a whey starter (NWS), from the previous day's processing.

4.The curd is then broken down by the master cheese-maker into small granules using a traditional tool called "spino"



4. The cooking process reaches 55°C degrees, after which the cheese granules sink to the bottom of the cauldron forming a single mass.

5.After about fifty minutes the cheese-maker removes the cheese mass by wrapping into the typical linen cloth and this give rise to two twin wheels, the cheese is then placed in a mould which give its final shape

6.After a few days, the wheels are immersed in a saturated water solution: it is a process of salting by osmosis. This last passage closes the production cycle of Parmigiano Reggiano and starts its maturation period.

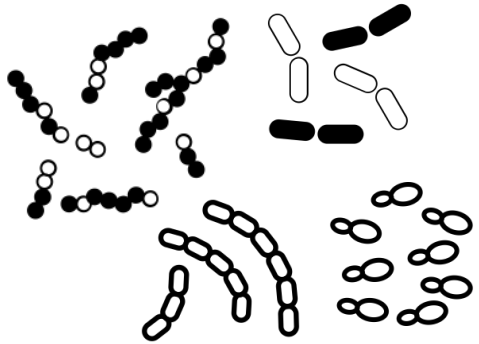
7. The maturation between 15-18°C and humidity around 80%, minimum ripening time is 12months, or up to 24, 36, 40 months and more.

Levante et al., 2021

Role of raw milk in cheesmaking

Raw milk is collected twice a day from each farmers, in the evening and in the morning.
Milk is collected and processed every day during the whole year as it cannot be stored

Raw milk carries so far underestimated biodiversity into the cheese



Complex interactions among starter lactic acid bacteria (SLAB) and nonstarter lactic acid bacteria (NSLAB)

SLAB

Acidification of the curd

NSLAB

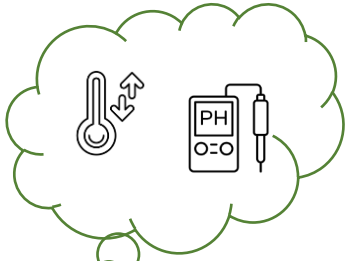
Ripending proteolysis

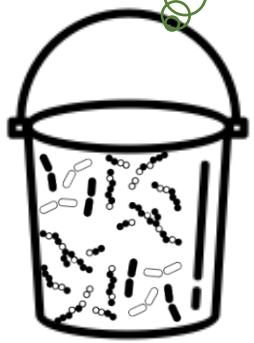
Where they originate?

Raw milk microbiota

Lab are not dominant species of raw milk microbiota

NSLAB

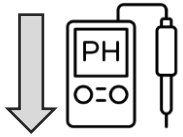


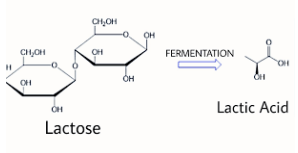


Natural whey starter (NWS)
Undefined whey starter cultures, dominant:

- *Lactobacillus helveticus*
- *Lactobacillus delbrueckii*
- *Limosilactobacillus fermentum*
- *Streptococcus thermophilus*
- *Lactococcus lactis*

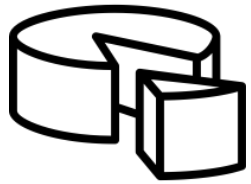
SLAB





SLAB

NSLAB



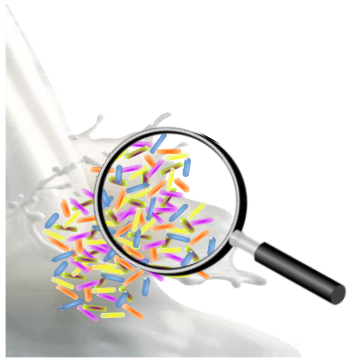
Gatti et al.,2014

Giving importance to raw milk lactic acid bacteria: one of the objectives of Farm4PR project

The importance of raw milk microbiota as a solid linkage to the territory, is in contrast with the current trend in Italy: raw milk is getting poorer in terms of microbial species, as a low microbial count is requested as a quality feature and milk is paid accordingly.

MINISTERO DELLE POLITICHE AGRICOLE ALIMENTARI E FORESTALI

Ministry of Agriculture, Forestry and Tourism



One of the main goal is to improve the quality based milk payment, considering LAB count beside total bacterial count

✓Description and classification of farming systems (feeding, environmental factors..)

✓Investigation of the natural aptitude of milk produced in the PR area to be fermented by lactic bacteria.

✓Evaluation of the dairy characteristics of the milk produced in the PR district.

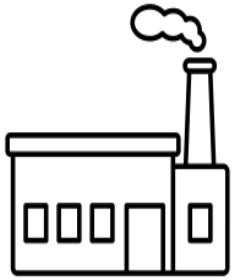
✓**Inclusion of innovative characteristics related to the quality of milk (e.g. LAB count) in the milk payment system of the PR district**

Expected outcomes

Relate the quality of raw milk to a new microbiological character: the presence of autochthonous lactic acid bacteria, as a consequence of the environmental and farming variables.

This aspect appears particularly important because transcending the classical total bacteria count, it could offer a new tool to describe the quality of milk, giving importance to raw milk lactic acid bacteria.

120 farmers



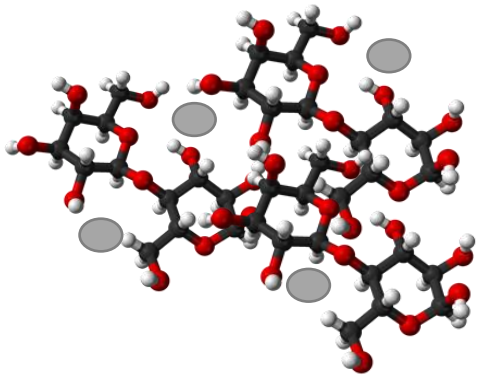
8 cheese factories

1 year sampling

1500 milk samples collected for microbiological analysis

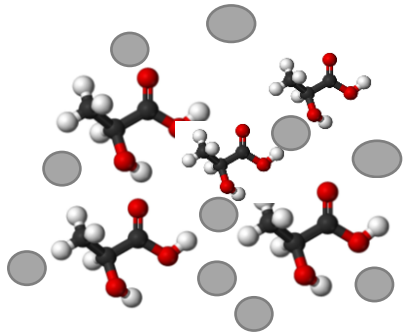
subjected to impedometric analysis to quantify LAB, focus on **NSLAB**

What is impedometric analysis???



Big uncharged molecules

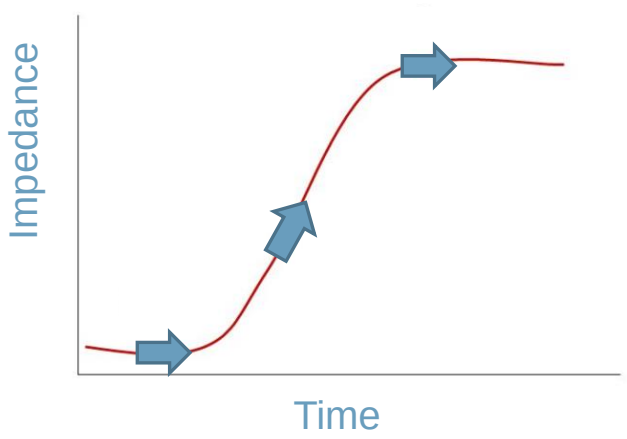
Microbial metabolism

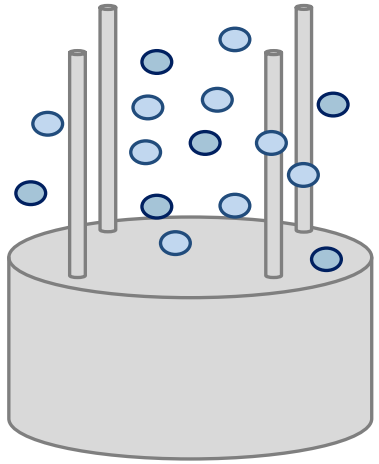


Smaller charged molecules

These changes in the molecular composition increase the conductivity of the liquid

Increase of the total **impedance**





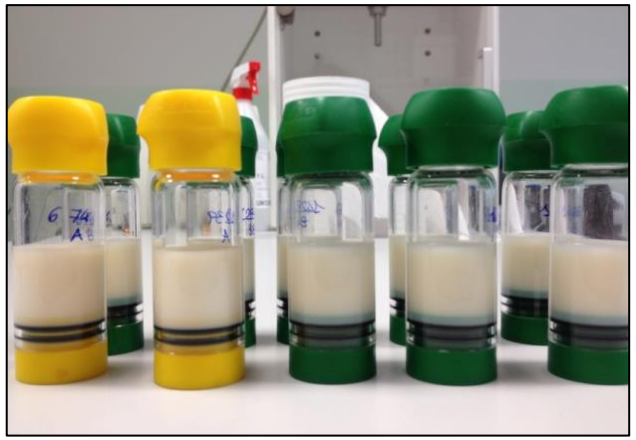
If this measurement are plotted against time, the microbial growth can be graphically represented

Bancalari E. et al., 2016 Frontiers in Microbiology

BacTrac 4300®
(SY-LAB, Neupurkersdof, Austria)

- The conventional M-value that corresponds to the media impedance (Conductance)
- E-value which is the electrochemical double layer of the electrodes-electrolyte impedance (Capacitance)
- Both these values, simultaneously recorded by the instrument, are shown as relative changes compared to a starting value and expressed as M% and E%

Incubators → 2 different temperatures

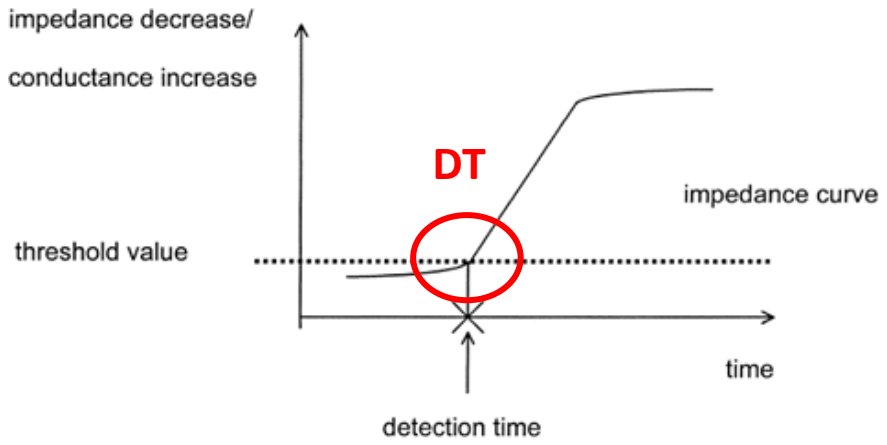


- 1500 samples in triplicated
- Raw milk (6ml) directly inside the vials
- Temperature: 25°C
- Real-time measurement
- Every 10 minutes one impedometric measurement

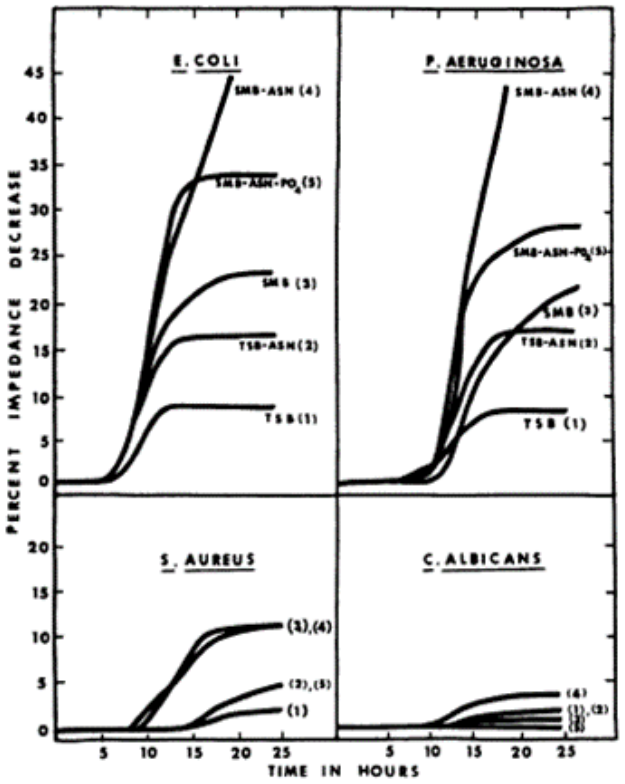
Bancalari E. et al., 2016 Frontiers in Microbiology

How the impedometric data can be interpreted?

The principle of this technique is not new, but its application in the microbiology field is more recent and is mainly associated to a rapid detection of foodborne pathogenic bacteria



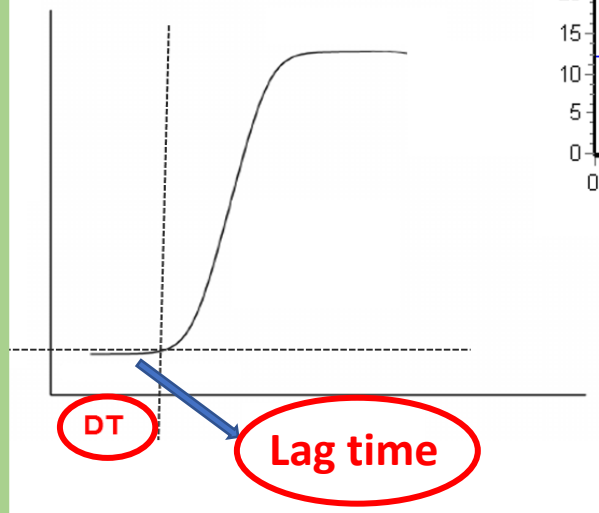
Wawerla, M., et al.,1999, *Journal of Food Protection*

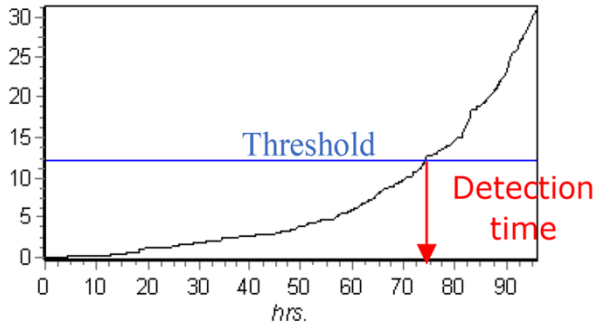


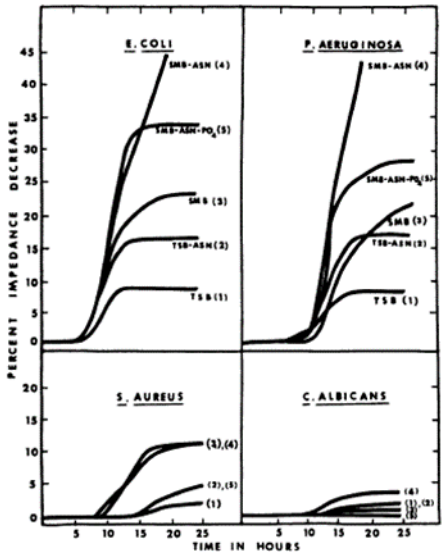
The most common way to use this curves in microbiology analysis, is to fix a point, generally defined as “time of detection” that coincides with a cell concentration about 10^6-10^7, or to compare the different curves obtained

Bancalari E. et al., 2016 and 2019

PAST

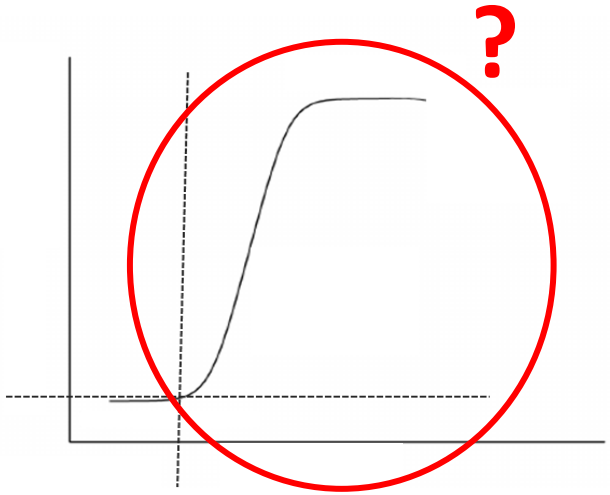






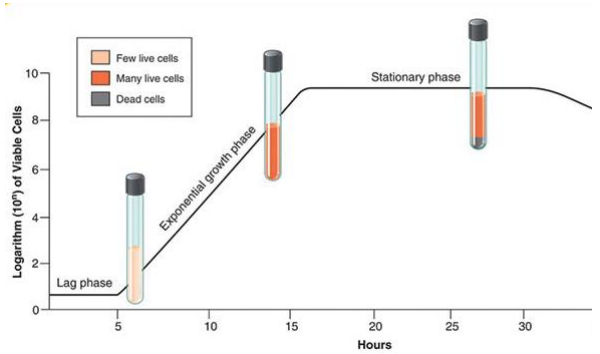
FUTURE

Can the rest of the curve be used to obtain useful informations?

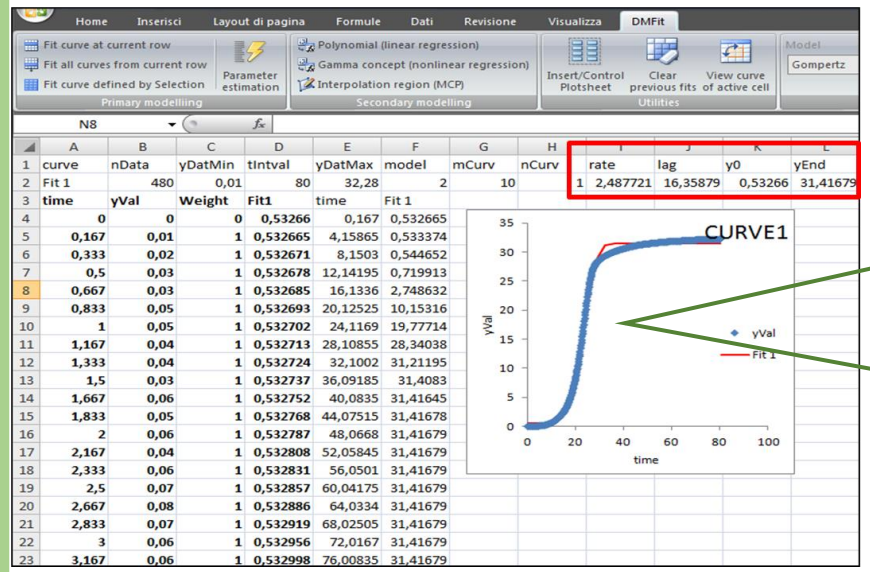


Use of predictive models for the impedometric data ????

Primary models describe how the number of microorganisms in a population changes with time under specific conditions

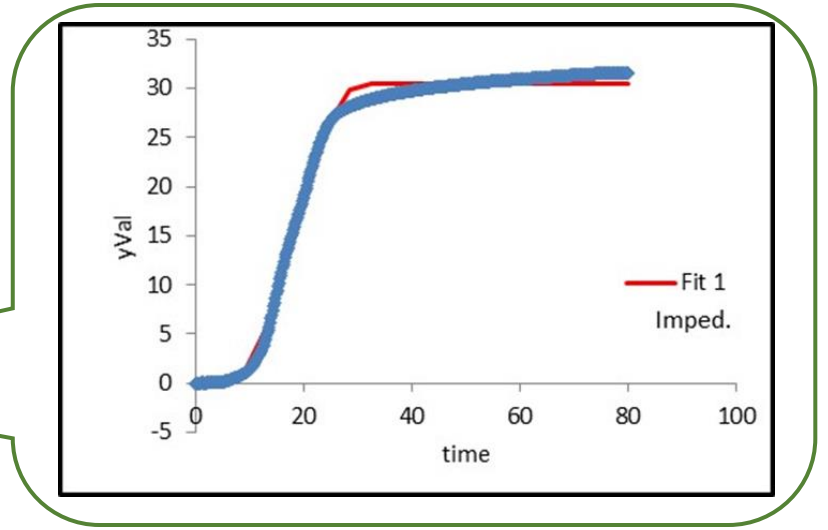


ComBase tools (Combase,Excel add-in, DMFit www.ifr.ac.uk/safty/DMFit)



curve	nData	yDatMin	tIntval	yDatMax	model	mCurv	nCurv	rate	lag	y0	yEnd
Fit 1	480	0,01	80	32,28	2	10	1	2,487721	16,35879	0,53266	31,41679

time	yVal	Weight	Fit1	time	Fit 1
0	0	0	0,53266	0,167	0,532665
0,167	0,01	1	0,532665	4,15865	0,533374
0,333	0,02	1	0,532671	8,1503	0,544652
0,5	0,03	1	0,532678	12,14195	0,719913
0,667	0,03	1	0,532685	16,1336	2,748632
0,833	0,05	1	0,532693	20,12525	10,15316
1	0,05	1	0,532702	24,1169	19,77714
1,167	0,04	1	0,532713	28,10855	28,34038
1,333	0,04	1	0,532724	32,1002	31,21195
1,5	0,03	1	0,532737	36,09185	31,4083
1,667	0,06	1	0,532752	40,0835	31,41645
1,833	0,05	1	0,532768	44,07515	31,41678
2	0,06	1	0,532787	48,0668	31,41679
2,167	0,04	1	0,532808	52,05845	31,41679
2,333	0,06	1	0,532831	56,0501	31,41679
2,5	0,07	1	0,532857	60,04175	31,41679
2,667	0,08	1	0,532886	64,0334	31,41679
2,833	0,07	1	0,532919	68,02505	31,41679
3	0,06	1	0,532956	72,0167	31,41679
3,167	0,06	1	0,532998	76,00835	31,41679



- Sigmoidal Fitted curve
- 3 parameters:

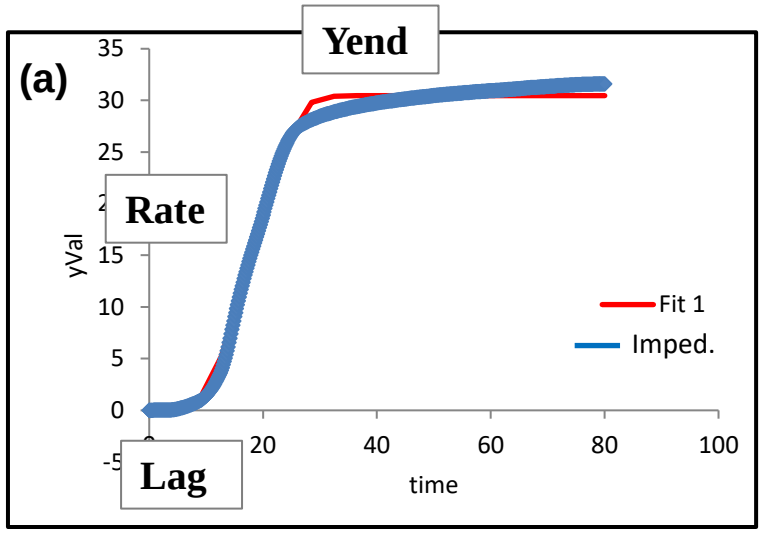
LAG
RATE
YEND

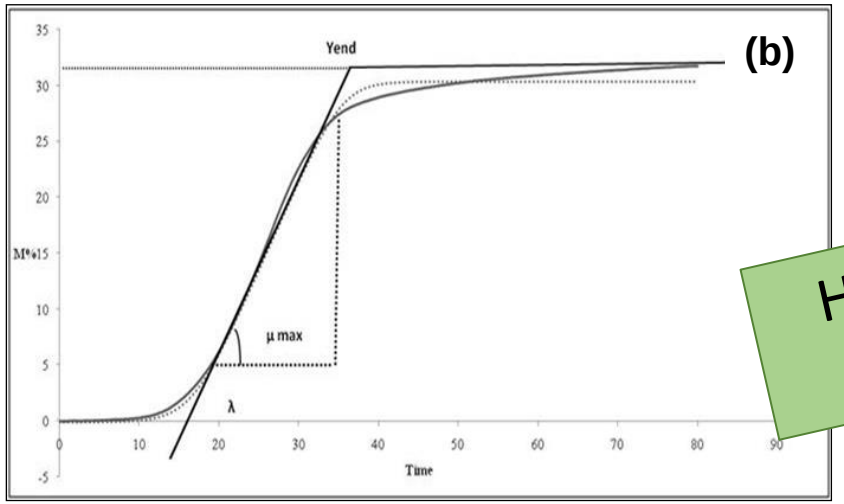
Blu line: original data
Red line: data fitted with the Gompertz equation

If curves overlap $R^2 \geq 0.9$, so the model works!!!

Bancalari E. et al., 2016 Frontiers in Microbiology

Impedometric fitted curve (a) microbial growth curve (b)





High throughput method

- **Lag time(λ) :** as Lag phase and is measured in hours (h)

This parameter measure the time that a population need to adapt to the growth conditions and start to grow
The bigger the Lag parameter, the higher the time that the cells need to start to grow

- Inoculum-dependent

- **Rate (µmax):** µmax that is directly correlated to the acidification rate, indeed, the higher the value the greater the acidification rate

- **Yend:** is the maximum value of impedance recorded and can be associated to the acidifying capability of the strains and the more the value, the greater the amount of acid produced

Bancalari E. et al., 2016 Frontiers in Microbiology

Applications

Acidyfing performances of starter lactic acid bacteria (SLAB)

- **Bancalari E**, Bernini V, Bottari B, Neviani E and Gatti M. Application of Impedance Microbiology for Evaluating Potential Acidifying Performances of Starter Lactic Acid Bacteria to Employ in Milk Transformation. (2016). Front. Microbiol. 7:1628.doi: 10.3389/fmicb.2016.01628

Probiotic and NSLAB growth behavior in food

- **Bancalari E,** Savo Sardaro ML, Levante A, Marseglia A, Caligiani A, Lazzi C, Neviani E, Gatti M. An integrated strategy to discover LAB strains for potential use as aromatic starter. Food Research International http://dx.doi.org/10.1016/j.foodres.2017.07.066.
- Prandi B, Baldassarre S, Babbar N, **Bancalari E,** Vandezande P, Hermans D, Bruggeman G, Gatti M, Elst K and Sforza S. Pectin oligosaccharides from sugar beet pulp: molecular characterization and potential prebiotic activity. (2018). Food and Function, 9:1557-1569. doi: 10.1039/c7fo01182b
- Paolo D'Incecco, **Elena Bancalari1**, Monica Gatti, Alessandro Ranghetti, Luisa Pellegrino. Low-temperature centrifugation of milk for manufacture of raw milk cheeses: impact on milk debacterization and cheese yield. LWT-Food Science and Technology
- Levante, A., **Bancalari, E.*,** Tambassi, M., Lazzi, C., Neviani, E., Gatti, M. (2020). Phenotypic diversity of *Lactobacillus casei* group isolate sas a selection criterion for use as secondary adjunct starters. (2020). Microorganisms; 17, 8:128. Doi: 10.3390/microorganisms8010128.
- **Bancalari, E**., Castellone, V., Bottari, B., Gatti, M. (2020). Wild *Lactobacillus casei* Group Strains: Potentiality to ferment plant derived juices. Foods. 9 (3), 14; Doi: https://doi.org/10.3390/foods9030314

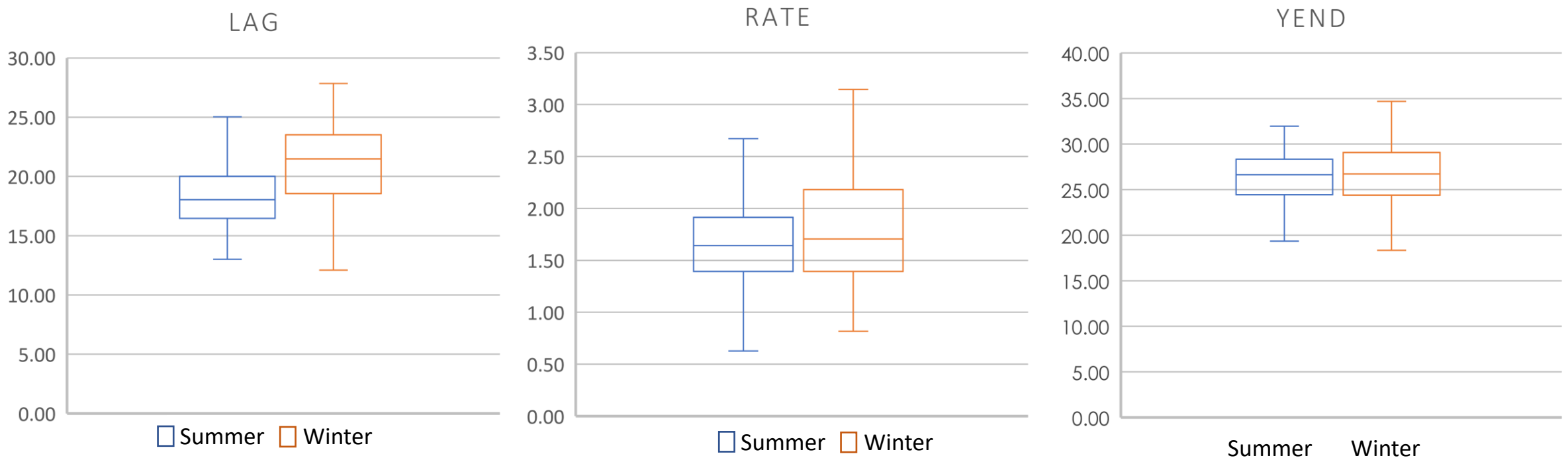
Study of Exopolysaccharides production by LAB

- **Bancalari E**, D'Incecco P, Savo Sardaro M.L., Neviani E, Pellegrino L., Gatti M. Impedance microbiology to speed up the screening of lactic acid bacteria exopolysaccharide production. (2019). doi: 10.1016/j.ijfoodmicro.2019
- Bancalari, E., Gatti, M., Bottari, B., Mora, M.,Arioli, S. (2022) Disclosing *Lactobacillus delbrueckii* subsp. *bulgaricus* intraspecific diversity in exopolysaccharides production. Food Microbiology 102 (2022) 103924

Effect of natural compounds on LAB and spoilage bacteria growth

- Martelli, F., Alinovi, M., Bernini, V., Gatti, M., **Bancalari, E.** (2020). Arthrospira platensis as natural fermentation booster for milk and soy fermented beverages. Foods; 18;9(3):350. Doi: 10.3390/foods9030350.
- **Bancalari, E*.,** Martelli, F., Bernini, V., Neviani, E., Gatti, M. (2020). Bacteriostatic or bactericidal? Impedometric measurement to test the antimicrobial activity of Arthrospira platensis extract. Food Control. Doi: https://doi.org/10.1016/j.foodcont.2020.107380
- **Bancalari, E*.,** Martelli, F., Bottari, B., Neviani, E., Gatti, M. (2021). Arthrospira platensis Extract: A Non-Invasive Strategy to Obtain Adjunct Attenuated Cultures. Foods doi: 10.3390/foods10030588

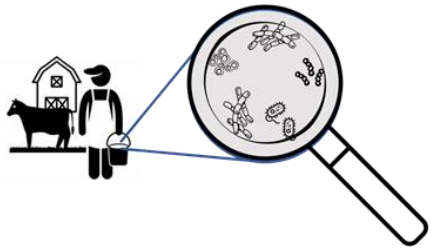
Preliminary results: Effect of the seasonality on the impedometric parameters

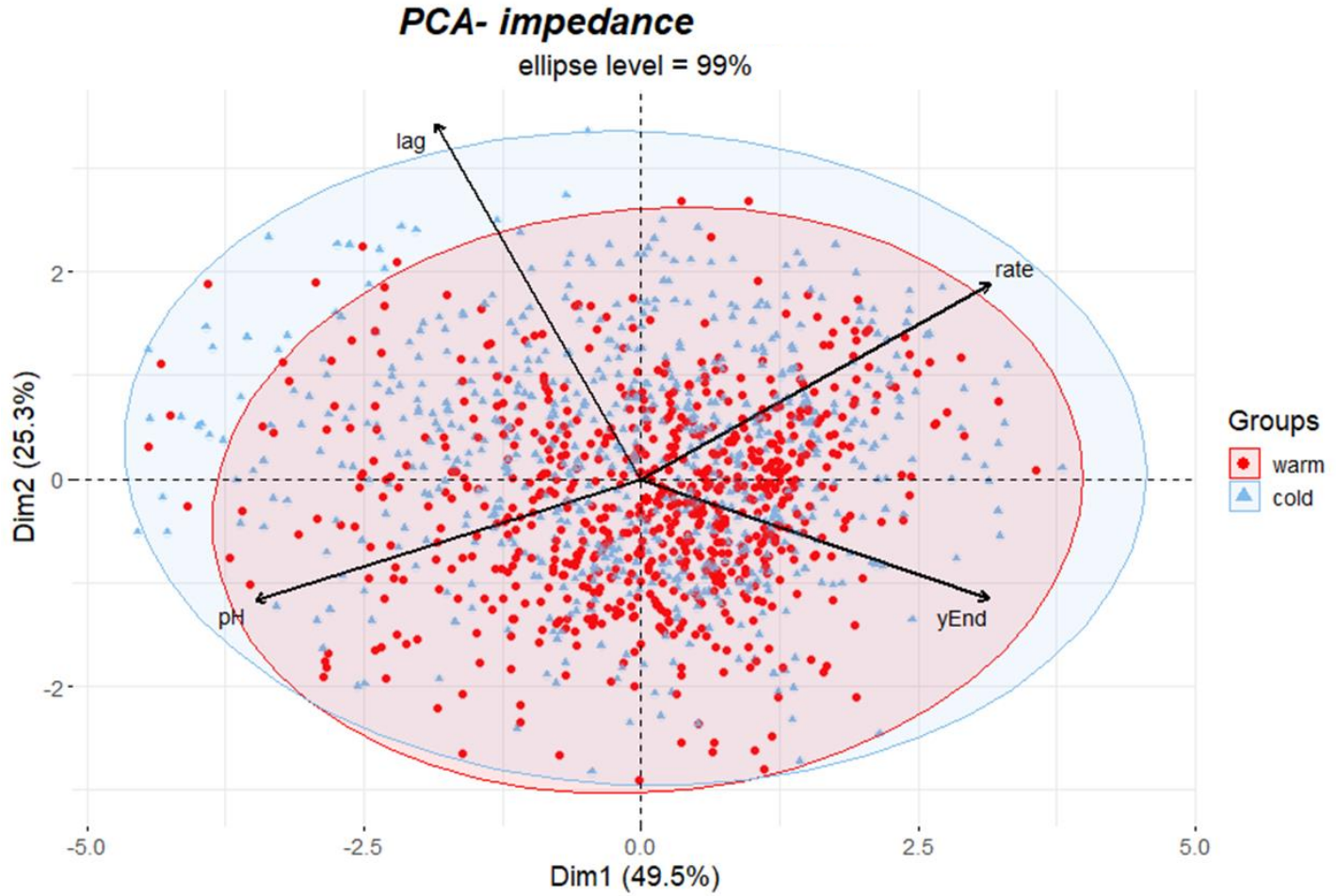


Mean values of Lag parameters of samples collected in summer are significantly lower the those collected in winter season ($p<0,05$)

Significantly lower Rate values in summer compared to winter samples ($p<0,05$)

Yend no statistically differences between summer and winter samples





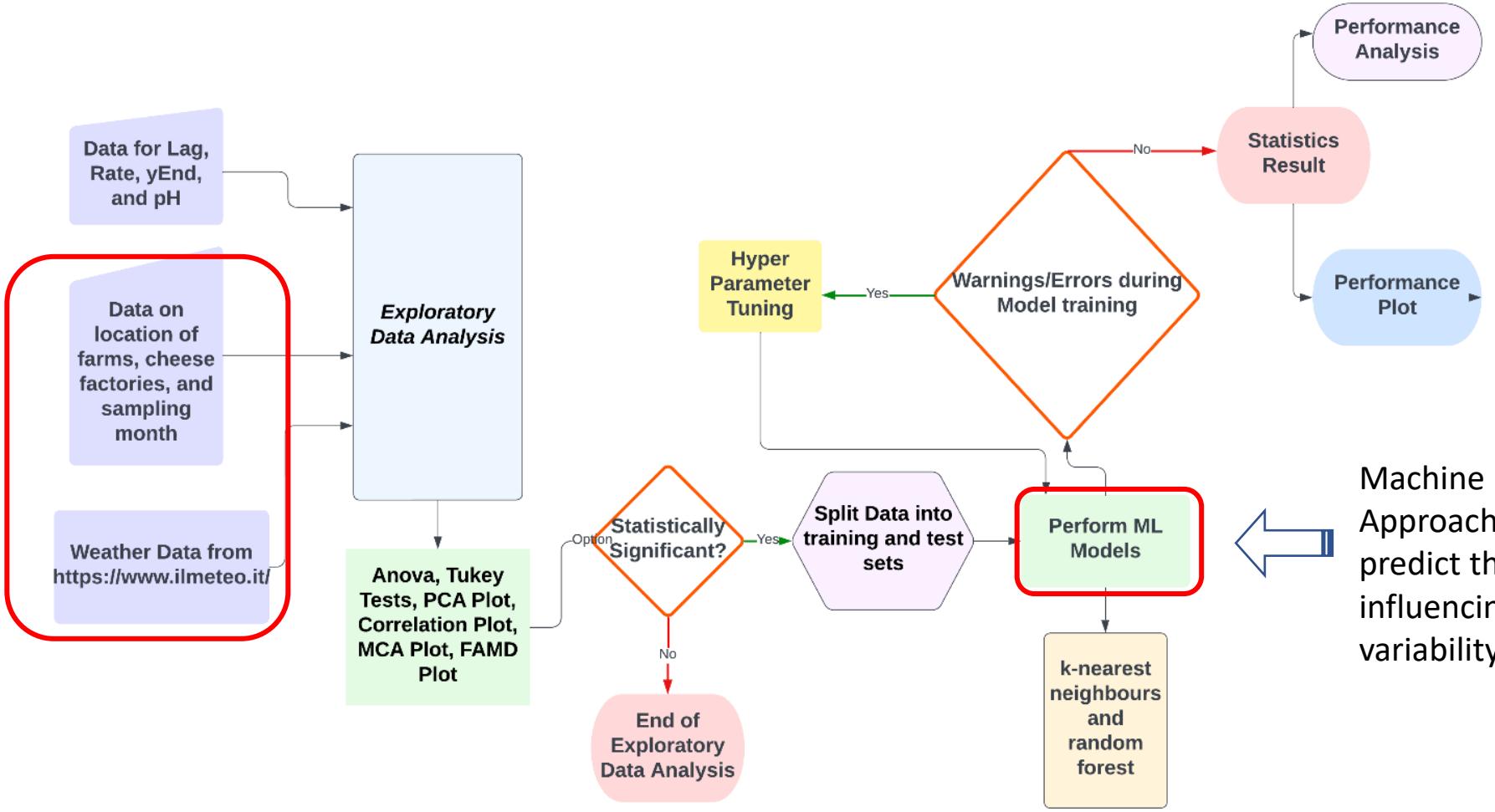
No distinct groups between the warm and cold season

Could other geographical factors, combined with the sampling months influence the parameters?

Machine Learning Approach to Examine the Factors Influencing the Non-Starter Lactic Acid Bacteria Population in Parmigiano Reggiano Cheese Production

Workflow

Further variable trying to better describe the variability within the NSLAB population

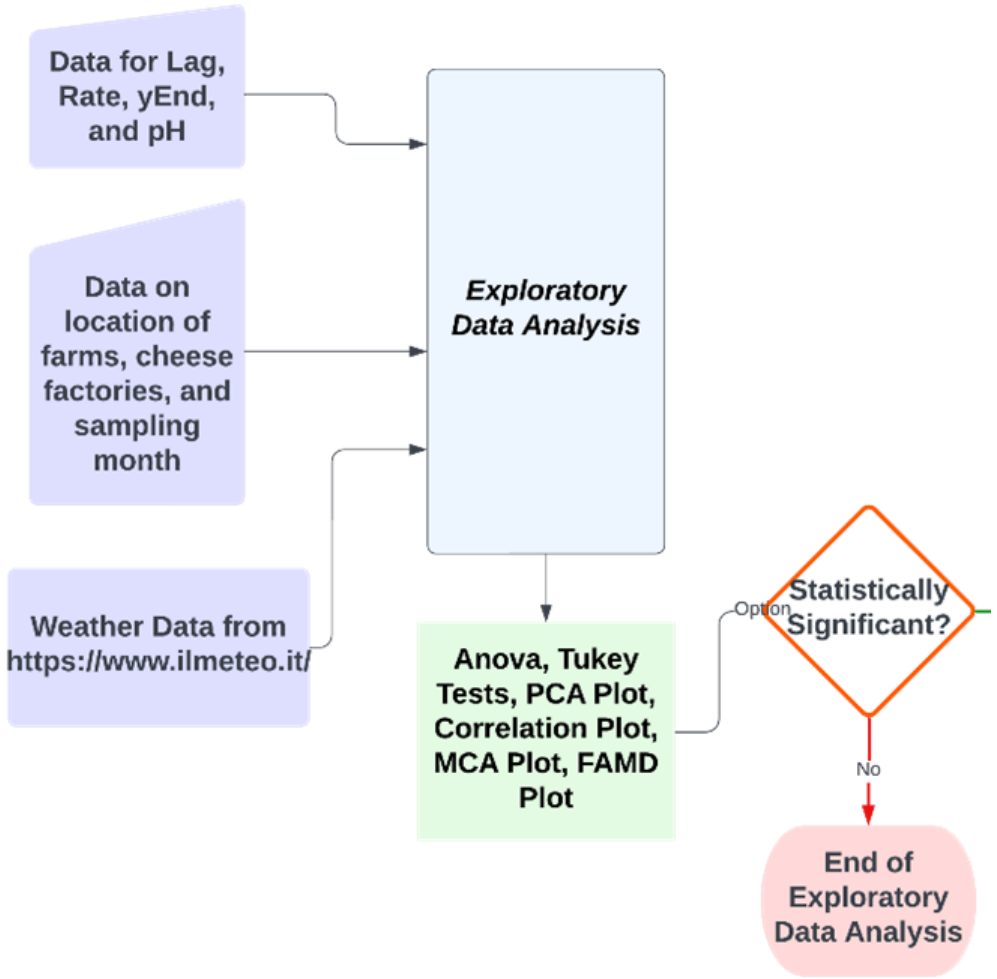


Machine Learning Approach trying to predict the factors influencing the variability of NSLAB

The weather and geographical factors were: temperature, range of altitude, maximum and median wind speed, dew point average pressure above sea level, visibility, humidity, and sampling month organized into season

Collaboration with Cranfield University

Results…



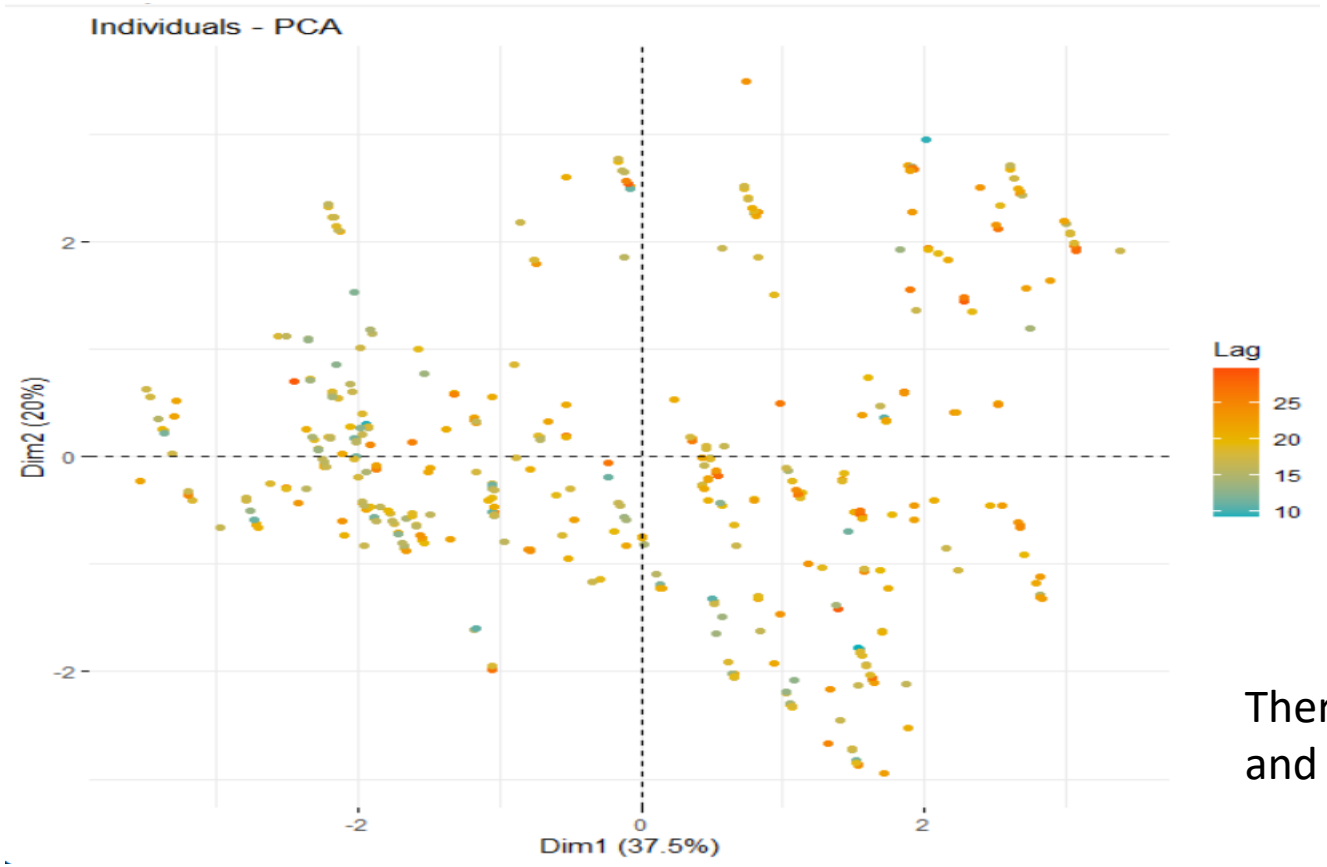
The results from the initial exploratory data analysis confirm the statistically significant differences of the parameters according to the two-way ANOVA test and MANOVA tests

Statistical Test	Lag p-value	Rate p-value	Maximum Rate (yEnd) p-value
Anova	Season: 2.0^-16 Cheese factory: 2.0^-16	Season: 0.0382 Cheese factory: 3.6^-14	Season: 0.0876 Cheese Factory:1.58^-6

This data are different enough to be used to predict this difference?

Giving the strogest significance of the differences, only Lag parameter will be uesed for the further tests

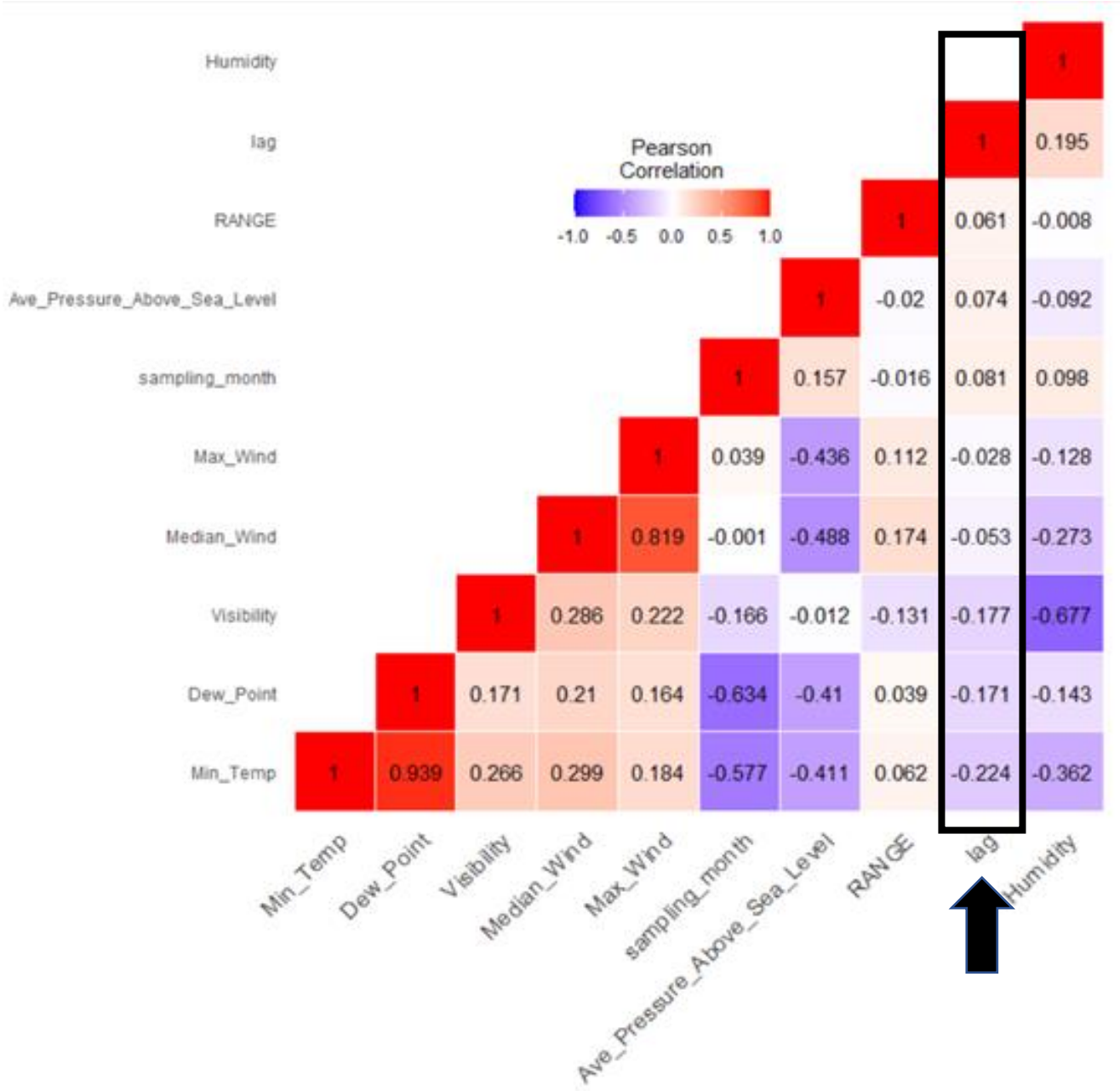
PCA plot of Minimum temperature, elevation (range), Maximum and Median Wind Speed, Humidity, Visibility, average pressure above sea level, and sampling month colored according to Lag.



Continuing with the exploratory data analysis, several multivariate analysis were used to visualize the possible correlations between the Lag and temperature related categories

There is no visible correlation between the weather and geographical factors and Lag

Pearson Correlation of weather and geographical factors of their correlation with Lag and to one another

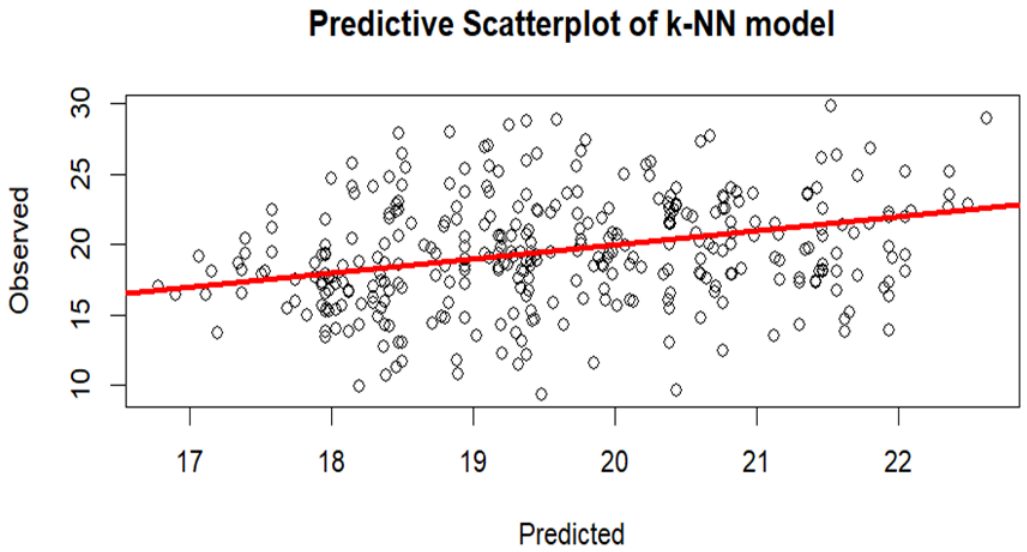


To further illustrate this lack of correlation with numbers, a correlation plot was made.

Lag is most correlated with the humidity and least correlated with the temperature

Lag also has a very minor correlation with pressure above sea level, sampling month, and range, that is range of altitude, but negatively correlated with every other continuous variable

A scatter plot of the Predicted and Observed Model as generated by the k-NN machine learning algorithm RMSE 3.77, $R^2 = 0.058$



Two algorithms were selected to build the machine learning models: Random Forest and k-nearest neighbours (k-NN)

A value of 3.93 demonstrates a high error between the predicted values based on the data the model was trained on, and the actual values of the test data.

This confirms that the weather variables cannot be utilized to predict the Lag, further supporting what was seen in the MCA, FAMD, correlation, and PCA plots

Other possible variable that could affect the number of different NSLAB but is not captured in the current dataset

Conclusion…

- ✓ High number of impedometric data giving useful information regarding NSLAB in raw milk
- ✓ Significative effect of seasonality on the impedometric parameters studied
- ✓ High number of data collected allowed us to apply a complex bioinformatic elaboration of the data
- ✓ The exploratory data analysis showed no clustering between the Lag and weather factors, as well as geographical factors
- ✓ The machine learning models were unable to accurately predict the Lag parameter, as evidenced by the high RMSE and low R^2 values from each model.
- ✓ The population of NSLAB in the raw milk sample cannot be predicted using the current data set.
- ✓ Interesting results: suggest that there are other possible factors that influence the NSLAB population in raw milk.

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