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(54) Title: A METHOD FOR TREATING TOBACCO MATERIAL AND TREATED TOBACCO MATERIAL

(57) Abstract: A method for treating tobacco material, the method comprising: providing a tobacco material, fermenting the tobacco material to obtain fermented tobacco material, the fermenting step including: # incubating the tobacco material under anaerobic conditions; # stopping the fermentation when at least one of the following conditions is satisfied: # the content of Lactic Acid is more than 10 times, preferably more than 20 times, more preferably more than 50 times, more preferably more than 70 times, preferably more than 80 times an initial amount of Lactic Acid in the tobacco material, # the content of Reducing Sugars is lower than 0.5, preferably lower than 0.4, more preferably below 0.2, more preferably below 0.1 an initial amount or Reducing Sugars in the tobacco material, # the content of Indole-3 Lactic Acid is more than 5 times, preferably more than 10 times, preferably more than 20 times, an initial amount of Indole-3 Lactic Acid in the tobacco material, # the content of caffeic acid is more than 4 times, preferably more than 10 times, preferably more than 20 times, an initial amount of caffeic acid in the tobacco material. # the content of quinic acid is more than 2 times, preferably more than 4 times, an initial amount of quinic acid in the tobacco material, # the content of asparagine is lower than 0.5, preferably lower than 0.4, preferably lower than 0.3 an initial amount or asparagine in the tobacco material, # the content of Glutamine is lower than 0.5, preferably lower than 0.4 an initial amount or Glutamine in the tobacco material, # the content of L-Ornithine is more than 10 times, preferably more than 20 times, preferably more than 50 times, preferably more than 100 times an initial amount of L-Ornithine in the tobacco material, # the content of L-Leucine is more than 2 times, preferably more than 4 times, an initial amount of L-Leucine in the tobacco material, # the content of L-Lysine is more than 2 times, preferably more than 6 times, an initial amount of L-Lysine in the tobacco material, # the fermentation index is more than 50, preferably more than 100, more preferably more than 250, more preferably more 400, wherein the fermentation index is obtained dividing the ratio between the content of Lactic Acid in the treated tobacco material and the content of Lactic Acid in the non-fermented tobacco material by the ratio between the content of Reducing Sugars in the treated tobacco material and the content of Reducing Sugars in the non-fermented tobacco material.

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A METHOD FOR TREATING TOBACCO MATERIAL AND TREATED TOBACCO MATERIAL

The present invention relates to a method for treating tobacco and a tobacco material treated by fermentation. The invention also relates to an aerosol generating article containing tobacco material treated by fermentation. In particular, the fermentation is an anaerobic fermentation.

Various treatment methods and additives have been proposed in the art for altering the overall character or nature of tobacco materials utilized in tobacco products. For example, tobacco materials have been treated with additives. In addition, treatment conditions used during the processing of those tobacco materials have been controlled, in order to alter the chemistry or sensory properties of tobacco products produced from such tobacco materials, and to alter the chemistry or sensory properties of mainstream smoke or aerosol generated by smoking articles incorporating such tobacco materials.

Treatments to enhance or add flavours and aromas to the tobacco material at a later stage of tobacco processing often involve the addition of one or more additive(s) to the tobacco and can require additional processing steps and equipment, which can be costly and time-consuming. Furthermore, the addition of additives to the tobacco may be not well perceived by some consumers.

There is therefore a need to provide a tobacco material having good and ameliorated organoleptic properties void of additives. There is also a need of a method for improving the organoleptic properties of a tobacco material which does not involve the addition of external flavourings to the tobacco itself. Furthermore, there is a need of a tobacco material which displays such different organoleptic material without requiring a complex processing.

There is also the need to provide a tobacco material having at the same time high organoleptic properties and a reduced concentration of harmful compounds. There is also the need to provide a tobacco material having at the same time high organoleptic properties and that produces when used by a user a reduced concentration of harmful compounds in the aerosol generated.

According to an aspect, the invention relates to a method for treating tobacco material, the method comprising providing a tobacco material and fermenting the tobacco material to obtain a fermented tobacco material. Preferably, the fermenting step includes: incubating the tobacco material under anaerobic conditions.

Due to the fermentation, certain chemical compounds present in the tobacco material may change and in turn organoleptic properties of the tobacco material may vary as well. Furthermore during the fermentation process the content of some chemical compounds in the tobacco material decreases and the concentration of some further chemical compounds increases.

The fermented tobacco material, at the sensory level, consistently delivers higher smoothness characters compared to the corresponding unfermented cured tobacco material. In addition fermented tobacco material delivers in use floral notes or scent notes. The tobacco fermented material has a smoother character reducing harshness sensation. Therefore, the organoleptic properties of the tobacco material are increased and the experience for the user is improved.

It is known that tobacco material may ferment. Tobacco plants may host microorganisms which in turn may include bacteria, mould and actinomyces. Studies have shown that bacteria occupy most of the present microorganisms in the tobacco, while mould and actinomyces are minorities. Yeast has a low concentration or cannot be detected at all. Fermented tobacco can be made by various suitable techniques known in the art, for example as described in "*Research Progress in Tobacco Fermentation*" published by Yang Yang et al, Journal of Biosciences and Medicines 2018, 6, 105-114 available on line at: http://www.scirp.org/journal/jbm; or in US 5372149, or in US 4528993, and others. In general, tobacco fermentation includes adjusting the moisture content of cured, aged tobacco to a moisture content of from about 20 percent to about 60 percent, and allowing the moistened tobacco to ferment in piles. Fermentation can be terminated for example by drying or cold storage. Tobacco fermentation does not need the addition of microorganisms because, as mentioned above, microorganisms are generally naturally present in the tobacco plants.

In the present invention the fermentation takes place under anaerobic condition.

Anaerobic fermentation is defined as the conversion of complex organic compounds into smaller molecules in the absence of oxygen. The term can be also defined as the conditions in which, as a result of both chemical equilibria and biochemical activities, oxygen is not available for redox reactions. Instead, other oxidized compounds may be present which can be used by micro-organisms for specific types of energy metabolism.

Anaerobic conditions may coexist with aerobic ones: oxygen in gaseous form may be unavailable to microorganisms in micro-environments (such as aggregates of detritus suspended in water) while at the same time it may be present in the macro-environment (water).

In tobacco anaerobic fermentation, without being bound by theory, the main energy extraction pathway may be coming from glycolysis, some amino acids being also used as carbon/nitrogen sources. The preferred nitrogenous compounds usually include glutamine, alanine, serine, threonine, aspartate, asparagine, urea, and arginine.

In the following, with the definition "fermentation conditions" it is meant that the tobacco is subjected to anaerobic conditions. A certain amount of oxygen may be present in the fermentation environment but Oxygen is not available for redox reactions.

The anaerobic fermentation conditions do not impact the content of alkaloids and in particular of nicotine of the tobacco material. At the same time, the organoleptic properties of the tobacco material are increased

Preferably the method provides for stopping the fermenting step when at least one desired condition is satisfied.

In this way it is possible to obtain a treated tobacco material having certain desired properties. And it is also possible to avoid too long fermentation processes. The quality of the tobacco material is therefore preserved.

Preferably, the method comprises stopping the fermenting step when the content of Lactic Acid is more than 10 times, preferably more than 20 times, more preferably more than 50 times an initial amount of Lactic Acid in the tobacco material. Preferably the method comprises stopping the fermentation when the content of Lactic Acid is more than 70 times, preferably more than 80 times, an initial amount of Lactic Acid in the tobacco material.

The increase of Lactic Acid is a reliable indicator of the fermentation of the tobacco material. The amount of the Lactic Acid in the tobacco material after fermentation may depend on the type of the tobacco material, or also on the initial amount of Lactic Acid in the tobacco material. Additionally the amount of Lactic Acid in the tobacco material after fermentation may depend on the content of other compounds in the tobacco material before fermentation. On the contrary, the increase of the Lactic Acid in the tobacco material is a reliable indicator of the degree of fermentation of the tobacco material. This parameter is not dependent on the type of the tobacco material or on the composition of the same, and it is not influenced by further external parameters.

Lactic Acid has two enantiomers L-Lactic Acid D-Lactic Acid. In the aim of the present invention with the term Lactic Acid it is intended the sum of two enantiomers L-Lactic Acid D-Lactic Acid. Nevertheless in fermented tobacco mostly the enantiomer L-Lactic Acid is present, however some D-Lactic Acid is also produced. Compared to L-lactic-acid, D-Lactic Acid can be toxic to human, i.e the LD50 value level per orally poisoned rats is around 4.5 g/kilograms (Pohanka, 2020). However, D-Lactic Acid and L-Lactic Acid are non-volatile, and therefore not transfer into the aerosol produced with the tobacco material.

The content of Lactic Acid in the tobacco material is determined by spectrophotometry, according to MP 0309 rev 5 2012, Chelab S.r.l.

Preferably the method comprises stopping the fermenting step when the content of Reducing Sugars is lower than 0.5, preferably lower than 0.4, more preferably below 0.2, more preferably below 0.1 an initial amount or Reducing Sugars in the tobacco material.

Reducing Sugars are converted by anaerobic fermentation to pyruvate, and pyruvic acid is a precursor of many other flavour compounds and provides caramel brown sugar and sour notes. Therefore, the decrease of the content of the Reducing Sugars in the tobacco material that is subjected to anaerobic fermentation is a good indicator of the degree of fermentation of the tobacco material. The amount of the Reducing Sugars in the tobacco material may depend on different factors, like for example the type of tobacco, the harvesting area, etc., but the reduction rate of the Reducing Sugars indicates the degree of the fermentation process. Most abundant naturally present sugars in tobacco leaves are glucose, fructose and sucrose. Differences in sugar content may be present among tobacco varieties. For example, Virginia has high level of sugar (generally in a range from 8 percent to 30 percent) while Burley is characterized with low content of sugars (generally in a range of 1 percent to 2 percent). However, regardless of the tobacco type which is used in the tobacco material, a reduction in the content of Reducing Sugars during the fermentation under the fermentation conditions of the invention has been found. Changes in the amount of Reducing Sugars may change the organoleptic properties of the tobacco material and of the smoke or aerosol produced with it.

Preferably the content of Reducing Sugars in the tobacco material is determined by continuous flow analyzer, according to CORESTA recommended method N° 38.

Preferably, the method comprises stopping the fermenting step when the content of Indole-3 Lactic Acid is more than 5 times, preferably more than 10 times, preferably more than 20 times, an initial amount of Indole-3 Lactic Acid in the tobacco material. Preferably the absolute content of indole-3 Lactic Acid was determined by UPLC-MS, calculated based on extracted standard curves.

The Indole-3 Lactic Acid is present only in traces in tobacco leaf but it is produced during anaerobic fermentation in cured leaf material and indicates that the tobacco material has been subjected to an anaerobic fermentation.

Preferably, the method comprises stopping the fermenting step when the content of caffeic acid is more than 4 time, preferably more 10 times, preferably more than 20 times, an initial amount of caffeic acid in the tobacco material.

The presence of the caffeic acid increases the organoleptical properties of the tobacco material improving the experience for the user. Preferably the content of caffeic acid in the tobacco material is estimated by metabolomic analyses using UPLC-MS, comparison of caffeic acid in non-fermented vs treated tobacco material, i.e. fermented or partly fermented tobacco material.

Preferably, the method comprises stopping the fermenting step when the content of quinic acid is more than 2 times, preferably more than 4 times, an initial amount of quinic acid in the tobacco material.

Preferably the content of quinic acid in the tobacco material is estimated by metabolomic analyses using UPLC-MS, comparison of caffeic acid in non-fermented vs treated tobacco material, i.e. fermented or partly fermented tobacco material.

Quinic acid and caffeic acid largely increased after the fermentation run as the result of cinnamoyl esterase catabolizing chlorogenic acid. The increase of these compounds does not depend on the tobacco substrate used for fermentation. Besides powerful antioxidant activity, increasing collagen production and prevention of premature aging, caffeic acid has demonstrated antimicrobial activity and may be promising in the treatment of dermal diseases (Magnani et al., 2014). Additionally, quinic acid is also a potent drug candidate to combat prostate cancer (Inbathamizh et al., 2013).

Preferably the method comprises stopping the fermenting step when the content of asparagine is lower than 0.5, preferably lower than 0.4, preferably lower than 0.3 an initial amount or asparagine in the tobacco material. Preferably the content of asparagine is determined by ion-exchange chromatography, according to MP 2442 rev 0 2021, Chelab S.r.l.

The reduction of asparagine implies a reduced conversion to acrylamide. Tobacco material contains a certain quantity of amino acids. The amino acids may contribute substantially to the level of certain components in the smoke or aerosol produced by the final product where the fermented tobacco material is contained, and to the sensory properties of the smoke or aerosol. Different type of tobaccos may contain different quantities of amino acids. Furthermore, there can be a difference in amino acid profile between tobacco leaf or tips or stalk, mainly of quantitative nature. Also, the growing location for the tobacco may alter the ratios of the levels of different amino acids, but rather similar profiles for the same tobacco amino acid are generally maintained. Regardless of the tobacco type and origin, during fermentation under the fermentation conditions of the invention, it has been observed that the asparagine content in the tobacco material decreases. This suggests that fermenting bacteria in the fermentation of the invention produce specific asparaginase(s) to assimilate C and N from amino acid resources. Asparagine may be converted thermally into acrylamide. Acrylamide is considered a potentially harmful substance. Decreasing the content of asparagine in the tobacco material allows obtaining a decrease in the acrylamide formation.

Preferably, the method comprises stopping the fermenting step when the content of Glutamine is lower than 0.5, preferably lower than 0.4 an initial amount or Glutamine in the tobacco material. Preferably the content of Glutamine is determined by ion-exchange chromatography, according to MP 2442 rev 0 2021, Chelab S.r.l.

The reduction of Glutamine allows the flavourings of the tobacco material to be increased since the reduction of Glutamine implies that an umami savory note is released form the tobacco material in use.

Preferably the method comprises stopping the fermenting step when the content of L-Ornithine is more than 10 times, preferably more than 20 times, preferably more than 50 times, preferably more than 100 times an initial amount of L-Ornithine in the tobacco material. Preferably the content of L-Ornithine is determined by ion-exchange chromatography, according to MP 2442 rev 0 2021, Chelab S.r.l.

The increase of L-Ornithine in the fermented tobacco material is a good indicator of the degree of fermentation of the tobacco material independently on the type of tobacco material subjected to fermentation and to the initial composition thereof. It has been found that independently on the type of tobacco material, the fermentation causes an increase of L-Ornithine in the tobacco material.

Preferably the method comprises stopping the fermenting step when the content of L-Leucine is more than 2 times, preferably more than 4 times, an initial amount of L-Leucine in the tobacco material. Preferably the content of L-Leucine is determined by ion-exchange chromatography, according to MP 2442 rev 0 2021, Chelab S.r.l.

Preferably the method comprises stopping the fermenting step when the content of L-Lysine is more than 2 times, preferably more than 6 times, an initial amount of L-Lysine in the tobacco material. Preferably the content of L-Lysine is determined by ion-exchange chromatography, according to MP 2442 rev 0 2021, Chelab S.r.l.

The increase of the L-Leucine and L-Lysine is a good indicator of the degree of fermentation of the tobacco material independently on the type of tobacco material subjected to fermentation and to the initial composition thereof.

Preferably the method comprises stopping the fermenting step when the content of 3-sec-Butylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione that is more than 20 times, preferably more than 40 times, more preferably more than 60 times, more preferably more than 80 times an initial amount of 3-sec-Butylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione in the tobacco material.

3-sec-Butylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione (BHHPPD) is detected only in traces in the cured tobacco material, but the content of BHHPPD in the tobacco material increases with the degree of fermentation independently from the type of tobacco material. Therefore, the increase of BHHPPD in the tobacco material imilligramss a good indicator of the degree of fermentation of the tobacco material.

Preferably the method comprises stopping the fermenting step when the content of Secoisolariciresinol (SECO) that is more than 10 times, preferably more than 20 times, more preferably more than 40 times, more preferably more than 50 times an initial amount of Secoisolariciresinol (SECO) in the tobacco material.

Only traces of Secoisolariciresinol are detected in the cured tobacco material, but the content of SECO in the tobacco material increases with the fermentation of the tobacco material independently from the type of tobacco material. Therefore, the increase of SECO in the tobacco material is a reliable indicator of the degree of fermentation of the tobacco material.

Preferably the method comprises stopping the fermenting step when the fermentation index is more than 50, preferably more than 100, more preferably more than 250, more preferably more 400. The fermentation index is obtained dividing the ratio between the content of Lactic Acid in the treated tobacco material and the content of Lactic Acid in the non-fermented tobacco material by the ratio between the content of Reducing Sugars in the treated tobacco material and the content of Reducing Sugars in the non-fermented tobacco material. The fermentation index may be obtained by the following formula: $Fr= (F_{LA}/NF_{LA}): (F_{RS}/NF_{RS})$ wherein: F_{LA}= content of Lactic Acid in the fermented tobacco material; NF_{LA} = content of Lactic Acid in the non- fermented tobacco material; F_{RS} = content of Reducing Sugars in the fermented tobacco material; NF_{RS} = initial content of Reducing Sugars in the non-fermented tobacco material. Also in this case Lactic Acid indicates the sum of L- and D- Lactic Acid, as indicated above.

The Fermentation index (Fr) allows also the little change in the composition of the tobacco material under fermentation to be detected. Moreover this parameter changes during the fermentation independently from the type of tobacco material under fermentation, therefore the parameter is highly reliable.

Stopping the fermenting step when at least one of the above conditions is satisfied allows obtaining a treated tobacco material having desired organoleptic properties. The tobacco material treated with the method of the invention could be fully fermented or partly fermented. Independently of the degree of fermentation of the tobacco material, by stopping the fermenting step when one of the above conditions is satisfied allows for obtain a tobacco material having certain features.

Treated tobacco material in the aim of the invention indicates fermented or partly fermented tobacco material.

Preferably the method further comprises an initial measuring step for measuring the initial content of at least one of Lactic Acid, or Reducing Sugars, or Indole-3 Lactic Acid, or quinic acid, or caffeic acid, or Asparagine, or Glutamine, or L-Ornithine, or L-Leucine, or L-Lysine, or Butylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione, or Secoisolariciresinol, or fermentation index in the tobacco material before the fermenting step so as to obtain an initial amount respectively of Lactic Acid, or Reducing Sugars, or Indole-3 Lactic Acid, or quinic acid, or caffeic acid, or L-Ornithine, or Asparagine, or Glutamine, or L-Leucine, or L-Lysine, or Butylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione, or Secoisolariciresinol (SECO) or fermentation index.

Owing to this feature it is possible to obtain a precise value of the initial content of at least one compound in the tobacco material before subjecting it to a fermentation process. Initial measuring step provides for measuring the features of the tobacco material before fermentation, in other words the features of the non-fermented tobacco material are measured in the initial measuring step. The initial amount of the measured features corresponds to the value of the feature in the non-fermented tobacco material.

Preferably the method further comprises an measuring step for measuring the content of at least one of Lactic Acid, or Reducing Sugars, or Indole-3 Lactic Acid, or quinic acid, or caffeic acid, or Asparagine, or Glutamine, or L-Ornithine, or L-Leucine, or L-Lysine, or Butylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione, or Secoisolariciresinol, or fermentation index in the tobacco material during fermentation.

Owing to this feature it is possible to obtain a precise value of at least one of the above indicated features in the tobacco material. It is possible to more precisely monitor the fermenting step and to more precisely adjust the properties of the tobacco material obtained. In this way it is possible to obtain in real time a very reliable indication of the degree of fermentation of the tobacco material.

Preferably, if the tobacco material contains dark tobacco, the method provides for stopping the fermenting step when the content of 2,3 butanediol is more than 5 times, preferably more than 10 times, an initial amount of 2,3 butanediol in the tobacco material. The presence of 2,3 butanediol confers a natural odor of cocoa butter. The presence of 2,3 butanediol contributes to a nice flavoured volatiles present in anaerobic fermented tobacco material.

Preferably, if the tobacco material contains dark tobacco, the method provides for stopping the fermenting step when the content of diacetyl is more than 5 times, preferably more than 10 times, an initial amount of diacetyl in the tobacco material. The presence of diacetyl confers some organoleptic properties to the tobacco material. The presence of diacetyl contributes to the presence of nice flavoured volatiles in anaerobic fermented tobacco material.

Preferably, the method comprises an initial measurement step for measuring the initial content of 2,3 butanediol or diacetyl in the tobacco material before the fermentation so as to obtain an initial amount respectively of 2,3 butanediol or diacetyl in the tobacco material.

Preferably, the method further comprises a measuring step for measuring the content of 2,3 butanediol or diacetyl in the tobacco material during fermentation.

Preferably, the method comprises providing a database containing the amount of at least one of Lactic Acid, or Reducing Sugars, or Indole-3 Lactic Acid, or quinic acid, or caffeic acid, or L-Ornithine, or Asparagine, or Glutamine, or L-Leucine, or L-Lysine, or Butylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione, or

Secoisolariciresinol (SECO), or fermentation index, or butanediol, or diacetyl in the non-fermented tobacco material.

Preferably the method comprises providing a database containing the amount of at least one of Lactic Acid, or Reducing Sugars, or Indole-3 Lactic Acid, or quinic acid, or caffeic acid, or L-Ornithine, or Asparagine, or Glutamine, or L-Leucine, or L-Lysine, or Butylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione, or Secoisolariciresinol (SECO), or fermentation index, or butanediol, or diacetyl in the non-fermented tobacco material for a plurality of different tobacco materials.

It is therefore possible to create at least one database for at least one type of tobacco material. Each database may contain the usual amount of Lactic Acid, or Reducing Sugars, or Indole-3 Lactic Acid, or quinic acid, or caffeic acid, or L-Ornithine, or Asparagine, or Glutamine, or L-Leucine, or L-Lysine, or Butylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione, or Secoisolariciresinol (SECO), or fermentation index, or butanediol, or diacetyl for each type of tobacco material in the non-fermented conditions.

Preferably, the method provides for retrieving an initial amount of at least one of Lactic Acid, or Reducing Sugars, or Indole-3 Lactic Acid, or quinic acid, or caffeic acid, or L-Ornithine, or Asparagine, or Glutamine, or L-Leucine, or L-Lysine, or Butylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione, or Secoisolariciresinol (SECO), or butanediol, or diacetyl, or fermentation index in the tobacco material before the fermentation from at least one database.

Preferably, the method comprises providing a database containing the amount of at least one of Lactic Acid, or Reducing Sugars, or Indole-3 Lactic Acid, or quinic acid, or caffeic acid, or L-Ornithine, or Asparagine, or Glutamine, or L-Leucine, or L-Lysine, or Butylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione, or Secoisolariciresinol (SECO), or fermentation index, or butanediol, or diacetyl in the fermented and/or partly fermented tobacco material.

Preferably the method comprises providing a database containing the amount of at least one of Lactic Acid, or Reducing Sugars, or Indole-3 Lactic Acid, or quinic acid, or caffeic acid, or L-Ornithine, or Asparagine, or Glutamine, or L-Leucine, or L-Lysine, or Butylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione, or Secoisolariciresinol (SECO), or fermentation index, or butanediol, or diacetyl in the fermented and/or partly fermented tobacco material for a plurality of different tobacco materials.

Preferably the method provides for retrieving from at least one database the amount of at least one of Lactic Acid, or Reducing Sugars, or Indole-3 Lactic Acid, or quinic acid, or caffeic acid, or L-Ornithine, or Asparagine, or Glutamine, or L-Leucine, or L-Lysine, or Butylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione, or Secoisolariciresinol (SECO), or fermentation index, or butanediol, or diacetyl and comparing the retrieved value with the corresponding measured value in the tobacco material under fermentation.

Preferably the method provides for stopping or continuing the fermenting step on the basis of the result of the comparing step. Preferably the methods provides for continuing the fermenting step if the measured value of Lactic Acid, or Indole-3 Lactic Acid, or quinic acid, or caffeic acid, or L-Ornithine, or L-Leucine, or L-Lysine, or Butylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione, or Secoisolariciresinol (SECO), or fermentation index, or butanediol, or diacetyl is lower than the corresponding retrieved value and/or the value of Reducing Sugars, or Asparagine, or Glutamine, or fermentation index is higher than the corresponding retrieved value.

According to another aspect the invention relates to a method for treating tobacco material, the method comprising providing a tobacco material and fermenting the tobacco material to obtain a fermented tobacco material. Preferably, the fermenting step includes: incubating the tobacco material under anaerobic conditions and stopping the fermenting step when at least one of the following conditions is satisfied:

- the tobacco material contains at least 20 milligrams per gram, preferably at least 50 milligrams per gram of Lactic Acid in total dry weight basis;
- the tobacco material contains less than 3 percent of total Reducing Sugars in total dry weight basis;
- the tobacco material contains less than 300 milligrams per kilogram of asparagine in total dry weight basis;
- the tobacco material contains less than 70 milligrams per kilogram of glutamine in total dry weight basis;
- the tobacco material contains more than 10000 milligrams per kilogram of asparagine in total dry weight of total free amino acids;
- the tobacco material contains more than 10 times, preferably more than 20 times, an initial amount of L-Ornithine in the tobacco material;
- the tobacco material contains containing more than 50 milligrams per kilograms, preferably more than 80 milligrams per kilograms, of L-Ornithine in total dry weight basis.

Preferably, the anaerobic conditions are achieved by placing the tobacco material in a container and closing the container. Preferably, the anaerobic conditions are achieved placing the tobacco material in a container, removing air from the container and closing the container. Preferably the container is not closed in a complete air-tight fashion, so that it is allowed for the CO_2 or other gases produced during fermentation escaping from the container. In this way, the accumulation of these substances in the container is avoided. Preferably the container is so isolated from the external environment to allow anaerobic conditions to be maintained inside the container. Preferably the container is closed with a closure avoiding an overpressure to be realised in the container.

More preferably, in order to remove the air from the tobacco material, pressure is applied. The applied pressure forces the air out from the tobacco material, so that, after the container is closed, oxygen is not present anymore, or it is present only in minimal quantity, in the container.

Placing the tobacco material in a container and closing the container, after the air has been removed from the closed container, allow reaching quickly the anaerobic conditions. This way of achieving anaerobic condition is preferable as it is cost effective and easy to implement.

Preferably, the fermenting step includes: applying a pressure to the tobacco material comprised between 1000 kilograms per square meter (Kilograms/m^2) and 15000 kilograms per square meter (Kilograms/m^2), preferably between 3000 kilograms per square meter and 12000 kilograms per square meter, more preferably between 5000 kilograms per square meter and 10000 kilograms per square meter.

The pressure applied to the tobacco material is maintained in the above range during the fermenting step.

The pressure may be applied to the tobacco material by any means. The pressure may be applied pumping an inert gas in the container. The pressure may be applied putting a weight on the tobacco material causing the desired pressure range to be applied to the tobacco material. For example, the container may be filled with wet tobacco material and, as a "lid" of the container, a weight is placed in contact to the tobacco material till water seeps out of the container. Preferably, the pressure is applied by means of a hydraulic actuator pressing on the closure of the container and/or on the tobacco material contained therein so as so establish the desired pressure in the container.

Preferably a hydraulic actuator is used for applying the desired pressure on the tobacco material. The hydraulic actuator may be chosen in dependence of the level of pressure to be realised in the container. Hydraulic actuators are known in the art for applying pressure to containers, such as for example wine presses. Preferably, the tobacco material is inserted in a container and a weight is located on top or above the tobacco material to exert the desired pressure. Preferably, then the container is closed, leaving the weight inside the container, so that the weight may keep applying pressure to the tobacco material.

Preferably, the fermenting step includes keeping the moisture content of the tobacco material during fermentation comprised between 10 and 50 percent in weight of the total weight of the tobacco material (weight by weight percentage). More preferably, the fermenting step includes keeping the moisture content of the tobacco material during fermentation comprised between 35 percent in weight and 45 percent in weight of the total weight of the tobacco material (weight by weight percentage). More preferably, the fermenting step includes keeping the moisture content of the tobacco material during fermentation at about 40 percent in weight (weight by weight percentage). After curing, the moisture of the tobacco material is generally low. Therefore, preferably water is added to the tobacco material to reach a desired moisture level.

More preferably, water is added also during the fermentation process in order to keep the moisture of the tobacco material comprised between 10 and 50 percent in weight , preferably 35 percent in weight and 45, more preferably about 40 percent in weight of the total weight of the tobacco material for at least one month, more preferably for at least 2 months, preferably for at least 6 months, even more preferably at least 8 months, preferably for at least 12 months, preferably for at least 24 months.

Preferably, in order to reach this moisture content, the tobacco material is wetted with water. Water is added to the tobacco material. Preferably, the tobacco material is wetted with water before being introduced in the container where the anaerobic conditions are created and kept.

Furthermore, during the fermenting step, this moisture content is maintained. Therefore, preferably, during the fermenting step, the moisture content of the tobacco material is monitored. For example, if the tobacco material is introduced in a container where the fermentation takes place, the container may be opened, and the moisture of the tobacco material may be measured when the container is opened. Preferably, the container is opened at regular intervals in order to perform the measurement of the tobacco moisture.

The moisture may be measured by a moisture sensor provided inside the container. In this way, the moisture may be measured also when the tobacco material is in the closed container.

Preferably, moisture of the tobacco material is measured at regular intervals.

Preferably, the fermenting step lasts for a fermentation time of at least 1 month, preferably at least 2 months, more preferably at least 4 months, more preferably at least 6 months, even more preferably at least 8 months, preferably at least 10 months. Preferably, the fermenting step lasts at least 12 months. Preferably, fermenting step lasts at least 24 months. Preferably, tobacco material is subjected to the fermentation conditions for a fermenting time less than 36 months, more preferably for a fermenting time less than 24 months. Preferably the fermenting step comprises a plurality of fermenting phases each fermenting phase lasting for a fermenting period. The overall fermenting step lasts for a fermentation time of at least 1 month, preferably at least 2 months, more preferably at least 4 months, more preferably at least 6 months, even more preferably at least 8 months, preferably at least 10 months, preferably at least 12 months, preferably at least 24 months.

The fermentation time may depend on the type of the tobacco material, or by the features of the tobacco material, or on at least one of the parameters of the fermenting step like for example Temperature, Pressure, Relative Humidity, etc..

The application of the fermentation conditions may be continuous for all the claimed time (for example, longer than one month, or longer than two months, or longer than 6 months, or longer than 12 months, or longer than 24 months). Alternatively, the fermenting step comprises a plurality of fermenting phases lasting

a certain fermenting period. In this case the fermentation conditions may be applied during a plurality of time intervals forming a sequence of time intervals. The different fermenting phases are separated one from the other by interruptions in which the fermenting conditions are not applied. It is considered an interruption a period of time in which one or more of the following conditions is interrupted: presence of anaerobic conditions, amount of moisture comprised between 25 percent in weight and 40 percent in weight of the total weight of the tobacco material, application of a pressure comprised between 1000 kilograms per square meter and 4000 kilograms per square meter. An interruption may take place in order check the tobacco material. For example, the moisture of the tobacco material may be measured during an interruption. An interruption may take place to turn or mix the tobacco material, so that a uniform treated tobacco material may be obtained. An interruption may last up to 6 hours.

The fermentation time is thus the total period during which the tobacco is subjected to the above fermentation conditions and it is to be calculated adding the duration of all the fermenting periods of each fermenting phases during which the fermentation conditions are indeed applied. Alternatively, the fermentation time can be calculated starting from the moment in which the fermentation conditions are applied for the first time and terminating when the fermentation conditions are applied for the last time, and then subtracting the duration of the interruptions.

For example, if a fermentation time T is selected, where with “fermentation time” the total period during which the tobacco material is subjected to the fermentation conditions is meant, the following cases are possible. The fermentation conditions are applied continuously for a total duration equal to T, in this case there is only one fermentation phase. A plurality of fermentation phases are provided and the fermentation conditions are applied for N fermentation periods: t_1, t_2, ..., t_N, where $t_1+t_2+...+t_N = T$. The time gap between a fermentation period t_j and the subsequent time interval t_{j+1} is the interruption. This total fermentation time T is of at least one month, or of at least two months, or of at least 6 months, or of at least 12 months, or of at least 24 months). Preferably, between two consecutive time intervals in which the fermentation conditions are applied, an interruption is present. The interruption does not last longer than 6 hours. Preferably, each fermenting phase lasts about 15 days, more preferably about 30 days. Preferably each fermenting phase lasts between about 30 days and about 60 days.

Preferably, the method provides for drying the tobacco material to obtain a dried tobacco material having a moisture content comprised between 5 percent and 10 percent in weight of the total weight of the tobacco material. The drying step is preferably carried out after the fermenting step under fermentation conditions has been terminated. After the fermenting step is terminated, the treated tobacco material is preferably removed from the container where it was placed, and the pressure applied to the tobacco is decreased. The treated tobacco material is then dried to a water content of between 1 percent and 15 percent in weight of

the total weight of the tobacco material, more preferably between 5 percent and 10 percent. The drying is performed so that the treated tobacco material may be easily processed is subsequent steps

Preferably, the method comprises the step of curing the tobacco material before the fermenting step. The tobacco material processed according to the method of the invention may comprise post-curing tobacco. As used herein, the term "post-curing tobacco" refers to tobacco that has been cured. The curing of the tobacco is preferably realized according to standard procedures and may depend on the type of tobacco which is included in the tobacco material. The tobacco material may include tobacco of different types and having had different curing. The tobacco of different types may be blended and then treated according to the invention.

Alternatively, the tobacco material is placed in a container and air is removed and replaced with water.

The container in which the tobacco material is placed is for example a barrel. Preferably, the barrel is made of wood, or concrete, or metal or a combination of any of these three materials.

The anaerobic conditions are kept for the desired duration of the fermenting step.

Preferably, the method provides for evaluating the colour of the tobacco material. The evaluation of the colour of the tobacco material may be performed also in addition to the measurement of a desired chemical substance. The colour of the tobacco material changes during the fermenting step. The evaluation of the colour of the tobacco material allows obtaining in an easy way an indication about the degree of fermentation.

Preferably, the method provides for stopping the fermenting step when the colour of the tobacco material reaches a desired colour. For example, the fermentation condition can be applied till the desired colour of the tobacco material is obtained. In this way, it is possible to improve the efficacy of the method of the invention.

Preferably the method provides for keeping the temperature of the tobacco material during the fermenting step comprised between 21 degrees Celsius and 35 degrees Celsius, preferably between 25 degrees Celsius and 31 degrees Celsius. Preferably, the temperature of the tobacco material during the fermenting step (while fermentation conditions are applied) remains comprised in a range of between 21 degrees Celsius and 35 degrees Celsius, more preferably between 25 degrees Celsius and 31 degrees Celsius. The temperature of the tobacco material is substantially maintained within this range during the whole fermenting step. The temperature is maintained by the fermentation itself, there is no need of providing or subtracting heat to the tobacco material. This temperature of the tobacco material during fermentation is obtained when the ambient temperature of the ambient where the tobacco material is located is preferably comprised between 15 degrees Celsius and 25 degrees Celsius.

Preferably, the method includes the step of turning the tobacco material, during the fermenting step. The turning of the tobacco material may provide an improved homogenization. Turning the tobacco material may means turning the tobacco material upside down. Turning the tobacco material may means overturning the tobacco material. The interruption of the fermentation conditions caused by the turning may be used also to measure certain parameters of the tobacco material, for example the moisture content. During the turning of the tobacco material, the fermentation conditions may be not applied any more. During the turning of the tobacco, the fermentation process may be interrupted, in the sense that the tobacco material may not be subjected to anaerobic condition during turning. After turning, preferably the fermentation conditions are re-applied to the tobacco material. Preferably the method includes the step of turning the tobacco material at a time interval of about 30 days. Preferably the method includes the step of turning the tobacco material at a time interval of about 15 days.

The different fermenting phases of the fermenting step are preferably interrupted by a turning step.

Preferably, the method comprises: securing the tobacco material within a moisture retaining material. This step of securing the tobacco material preferably takes place before the tobacco material is subjected to the fermentation conditions. It is desirable for the moisture-retaining material to be resistant to degradation during the tobacco treatment process (the fermentation). The moisture-retaining material may comprise a flexible material. This flexible material may be wrapped around the tobacco material. The moisture retaining material preferably comprises plastic material. Alternatively, or in addition, the moisture-retaining material may comprise a rigid material. The container in which the tobacco material is introduced may function as a moisture retaining material. In this case, the material of the container may include for example metal, wood, plastic, or concrete.

Preferably the method further comprises mixing a certain quantity of non-fermented tobacco material with a desired quantity of fermented or partly fermented tobacco material to obtain a tobacco material in which the amount of the fermented tobacco material in the tobacco material is comprised between 5 percent in weight and 10 percent in weight of the total tobacco material. The mixing step is preferably provided before the fermenting step. Preferably, it is provided for subjecting the tobacco material to the fermenting step after the mixing step.

This would improve the efficiency of the fermentation process, reducing the time required for the fermenting step and improving the characteristics of the fermented tobacco material obtained from the fermenting step.

Preferably the method comprises a cutting step in which the tobacco material is cut in pieces having dimensions comprised between about 0.3 millimeters and about 1.4 millimeters. Preferably the cutting step is performed before the fermenting step. This would improve the efficiency of the fermenting step. This

would also reduce the duration of the fermenting step, i.e. the time required for obtaining the desired changes of composition of the tobacco material.

Preferably the method further provided for maintaining the pH of the tobacco material during fermentation comprised between 4.5 and 5.5, preferably about 4.8 and 5.4. Preferably, the treated tobacco material is at least 100 times more acidic than the untreated tobacco material. The pH of the treated tobacco material and the pH of the untreated tobacco material may differ of at least 2 pH units. In different tobacco material, the pH may remain substantially unchanged.

Alternatively, or in addition, the tobacco material treated according to the method of the invention may comprise tobacco that has been re-graded, green-leaf blended, conditioned, de-stemmed or threshed (or not in the case of whole leaf), dried or packed.

According to another aspect, the invention relates to a tobacco material obtained by a process comprising fermenting the tobacco material to obtain treated tobacco material, the method including incubating the tobacco material under anaerobic conditions.

Preferably, the treated tobacco material comprises Lactic Acid. Preferably, the treated tobacco material has a content of Lactic Acid that is more than 10 times, preferably more than 20 times, more preferably more than 50 times, more preferably more than 70 times, more preferably more than 80 times, an initial amount of Lactic Acid in the tobacco material. In anaerobic fermentation, Lactic Acid is known to be a related catabolic product. The Lactic Acid may have a “smoothing effect” regarding nicotine harshness. The Lactic Acid may be responsible in the lowering of the pH of the treated tobacco material.

Preferably, the treated tobacco material has a content of Reducing Sugars that is lower than 0.5, preferably lower than 0.4, more preferably below 0.2, more preferably below 0.1 an initial amount or Reducing Sugars in the tobacco material. Preferably, the treated tobacco material comprises an amount of total Reducing Sugars at least 50 percent, more preferably 60 percent, even more preferably 85 percent lower than the amount of total Reducing Sugars contained in the same tobacco material before the treatment according to the method of the previous aspect. Preferably, at the end of the fermenting step, the amount of Reducing Sugars is at least 50 percent, more preferably 60 percent, even more preferably 85 percent lower than the amount of Reducing Sugars contained in the same tobacco material before the treatment. Reducing Sugars are the sum of the following: glucose, fructose, sucrose, maltose. The majority of the Reducing Sugars in the treated tobacco material may be converted. Reducing Sugars resources such as glucose and fructose present in the starting tobacco material may be used as a source of energy by the anaerobic bacteria. In the absence of oxygen, the glycolysis pathway transforms glucose (or fructose) into pyruvate. The altered levels of these compounds may contribute to the desirable taste and aroma of the treated tobacco material.

Preferably, the treated tobacco material has a content of Indole-3 Lactic Acid that is more than 5 times, preferably more than 10 times, preferably more than 20 times, an initial amount of Indole-3 Lactic Acid in the tobacco material.

Preferably the treated tobacco material has a content of caffeic acid that is more than 4 times, preferably more than 10 times, an initial amount of caffeic acid in the tobacco material.

Preferably the treated tobacco material has a content of quinic acid that is more than 2 times, preferably more than 4 times, an initial amount of quinic acid in the tobacco material.

Preferably the treated tobacco material has a content of asparagine that is lower than 0.5, preferably lower than 0.4, preferably lower than 0.3 an initial amount or asparagine in the tobacco material.

Preferably the treated tobacco material has a content of Glutamine that is lower than 0.5, preferably lower than 0.4 an initial amount or Glutamine in the tobacco material.

Preferably the treated tobacco material has a content of L-Ornithine that is more than 10 times, preferably more than 50 times, preferably more than 100 times an initial amount of L-Ornithine in the tobacco material.

Preferably the treated tobacco material has a content of L-Leucine that is more than 2 times, preferably more than 6 times, an initial amount of L-Leucine in the tobacco material in the tobacco material.

Preferably the treated tobacco material has a content of L-Lysine that is more than 2 times, preferably more than 6 times, an initial amount of L-Lysine in the tobacco material.

Preferably the treated tobacco material a fermentation index that is more than 50, preferably more than 100, more preferably more than 250, more preferably more 400. The fermentation index is obtained dividing the ratio between the content of Lactic Acid and an initial content of Lactic Acid in the tobacco material by the ratio between the content of Reducing Sugars and an initial content of Reducing Sugars in the tobacco material.

According to a further aspect the invention relates to a tobacco treated material containing at least one of the following features:

- Lactic Acid in an amount that is more than 10 times, preferably more than 20 times, more preferably more than 50 times, more preferably more than 70 times, more preferably more than 80 times, an initial amount of Lactic Acid in the tobacco material.
- Reducing Sugars in an amount that is lower than 0.5, preferably lower than 0.4, more preferably below 0.2, more preferably below 0.1 an initial amount or Reducing Sugars in the tobacco material,

- Indole-3 Lactic Acid in an amount that is more than 5 times, preferably more than 10 times, preferably more than 20 times, an initial amount of Indole-3 Lactic Acid in the tobacco material.
- caffeic acid in an amount that is more than 4 times, preferably more than 10 times, an initial amount of caffeic acid in the tobacco material,
- quinic acid in an amount that is more than 2 times, preferably more than 4 times, an initial amount of quinic acid in the tobacco material,
- asparagine in an amount that is lower than 0.5, preferably lower than 0.4, preferably lower than 0.3 an initial amount or asparagine in the tobacco material,
- Glutamine in an amount that is lower than 0.5, preferably lower than 0.4 an initial amount or Glutamine in the tobacco material,
- L-Ornithine is more than 10 times, preferably more than 50 times, preferably more than 100 times an initial amount of L-Ornithine in the tobacco material,
- L-Leucine in an amount that is more than 2 times, preferably more than 4 times, an initial amount of L-Leucine,
- L-Lysine in an amount that is more than 2 times, preferably more than 6 times, an initial amount of L-Lysine,
- a fermentation index is more than 50, preferably more than 100, more preferably more than 250, more preferably more 400, wherein the fermentation index is obtained dividing the ratio between the content of Lactic Acid in the tobacco material and the content of Lactic Acid in the non-fermented tobacco material by the ratio between the content of Reducing Sugars in the tobacco material and the content of Reducing Sugars in the non-fermented tobacco material.

The treated tobacco material having one or more of the previous indicated parameters has improved organoleptic properties. The above indicated parameters allow evaluating the quality and the possible appreciation of the tobacco material form a user. Additionally the above indicated parameters affect the organoleptic properties and thus the final taste of the tobacco material. Therefore it is possible, on the basis of the values of the above indicated parameters, to foreseen the group of users to which the tobacco material is preferably directed. Based on the values of one or more of the indicated parameters it is in fact possible to foresee which users will appreciate more the tobacco material.

Preferably the treated tobacco material has a content of 3-sec-Butylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione that is more than 20 times, preferably more than 40 times, more preferably more than 60 times, more preferably more than 80 times an initial amount of 3-sec-Butylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione in the tobacco material.

Preferably the treated tobacco material has a content of Secoisolariciresinol (SECO) that is more than 10 times, preferably more than 20 times, more preferably more than 40 times, more preferably more than 50 times an initial amount of Secoisolariciresinol (SECO) in the tobacco material.

According to another aspect the invention relates to a tobacco material containing at least 20 milligrams per gram, preferably at least 50 milligrams per gram of Lactic Acid in total dry weight basis.

According to another aspect the invention relates to a tobacco material comprising less than 3 percent of total Reducing Sugars in total dry weight basis.

According to another aspect the invention relates to a tobacco material comprising less than 300 milligrams per kilogram of asparagine in total dry weight basis.

According to another aspect the invention relates to a tobacco material comprising less than 70 milligrams per kilogram of glutamine in total dry weight basis.

According to another aspect the invention relates to a tobacco material comprising more than 10000 milligrams per kilogram of asparagine in total dry weight of total free amino acids.

According to another aspect the invention relates to a tobacco material comprising more than 10 times, preferably more than 20 times, an initial amount of L-Ornithine in the tobacco material.

According to another aspect the invention relates to a tobacco material containing more than 50 milligrams per kilograms, preferably more than 80 milligrams per kilograms, of L-Ornithine in total dry weight basis.

The tobacco material of any one of the previous aspects may be obtained by a process comprising fermenting the tobacco material, the method including incubating the tobacco material under anaerobic conditions.

The tobacco material is preferably cured. The tobacco material is preferably cured before being subjected to the fermenting step

According to another aspect of the invention it is provided Dark tobacco material having a content of 2,3 butanediol that is more than 5 times, preferably more than 10 times an initial amount of 2,3 butanediol in the tobacco material.

According to another aspect of the invention it is provided Dark tobacco material having a content of diacetyl that is more than 5 times, preferably more than 10 times an initial amount of diacetyl in the tobacco material.

According to another aspect of the invention it is provided Virginia tobacco material as tobacco material.

According to a further aspect of the invention it is provided an aerosol generating article comprising a tobacco material containing between about 2.5 percent by weight in total dry weight basis and 100 percent by weight

in total dry weight basis, preferably at least about 4 percent by weight in total dry weight basis, preferably at least about 10 percent by weight in total dry weight basis. The percentages are indicated by weight in total dry weight basis of a tobacco fermented material according to any one of the previous aspects.

According to a further aspect of the invention it is provided an aerosol generating article containing at least 5 milligrams per gram, preferably 10 milligrams per gram in total dry weight basis of Lactic Acid.

According to another aspect of the invention it is provided an aerosol generating article containing less than 300 milligrams per kilogram of asparagine in total dry weight basis.

According to another aspect of the invention it is provided an aerosol generating article containing less than 70 milligrams per kilogram of glutamine in total dry weight basis.

According to another aspect of the invention it is provided an aerosol generating article containing more than 10000 milligrams per kilogram of asparagine in total dry weight of total free amino acids.

Advantages of the tobacco material of the invention have been already outlined with reference to the previous aspects and not repeated herewith.

Preferably, the tobacco material includes hand – stripped leaves from which ribs have been removed.

Preferably, the tobacco material comprises Kasturi tobacco.

Preferably the tobacco material comprises Virginia tobacco.

Preferably the tobacco material comprises Dark tobacco.

The treated tobacco according to the method of the invention may change its chemical composition with respect to the untreated tobacco. “Treated tobacco material” in the present context means tobacco material that underwent the treatment as described in the previous process, that is, tobacco material that has been subjected for at least one month to the fermentation conditions. “Untreated tobacco material” in the present context means tobacco material that did not undergo the treatment as described in the previous method, that is, tobacco material that has not been subjected to the fermenting step. Untreated tobacco material is for example the tobacco material which is inserted in the container before the treatment of the invention starts. The treated tobacco material is compared with the same tobacco material which did not undergo the treatment according to the invention (the untreated tobacco material). The decrease in asparagine may be associated with an increase in aspartate. This suggests that fermenting bacteria produce specific asparaginase(s) to assimilate C and N from amino acid resources. This reaction may produce ammonia.

Non fermented tobacco material in the present context means tobacco material that did not undergo the treatment as described in the previous method, that is, tobacco material that has not been subjected to the

fermenting step. Fermented or partly fermented tobacco material in the present context means tobacco material that underwent the treatment as described in the previous method that is, tobacco material that has been subjected to the fermenting step for a certain period of time.

As used herein, the terms “change” or “changed” are used in the context of the flavour or organoleptic properties to mean that there is a modification from one overall taste or sensory character to another, as identified by expert smokers. This may include an improvement.

In the aim of the present disclosure, with the word “initial” it is considered the amount of a chemical substance or in general the value of a parameter of the tobacco material before subjecting the tobacco material to the fermenting step. In this sense the word initial may be considered synonymous of non-fermented.

As used therein the definition “fermenting step” or “fermentation” or “fermentation conditions” all indicate that the material is subjected for a desired extent of time to conditions suitable for causing the fermentation of the material.

The term "tobacco material" refers to any part of a tobacco plant or a mixture of different tobacco plants and includes without limitation tobacco leaf scraps, tobacco green leaf scraps, tobacco stems, tobacco dust created during tobacco processing, and tobacco leaf prime lamina strip and a combination thereof. The tobacco material can have the form of processed tobacco parts or pieces, cured and aged tobacco in essentially natural lamina or stem form, a tobacco extract or a mixture of the foregoing, for example, a mixture that combines extracted tobacco pulp with granulated cured and aged natural tobacco lamina. The tobacco material can be in solid form, in liquid form, in semi-solid form, or the like. Preferably, the term “tobacco material” includes any part and any related by-product, such as for example the leaves or stems, of any member of the genus *Nicotiana.* The tobacco material for use in the present invention is preferably from the species *Nicotiana tabacum.* Any type, style or variety of tobacco may be treated. Examples of tobacco which may be used include but are not limited to Virginia, Burley, and Oriental tobaccos, and blends of any of these types. Preferably, the tobacco material comprises Kasturi tobacco. The tobacco material to be treated may comprise or consist of post-curing tobacco.

As used herein, the term “post-curing tobacco” refers to tobacco that has been cured but has not undergone any further treatment process to alter the taste or aroma of the tobacco material. The post-curing tobacco may have been blended with other styles, varieties or types of tobacco. Alternatively, or in addition, the tobacco material to be treated may comprise or consist of tobacco that has been re-graded, green-leaf blended, conditioned, de-stemmed or threshed (or not in the case of whole leaf), dried or packed.

Preferably, the tobacco material comprises lamina tobacco material. The tobacco may comprise between about 70percent and 100percent lamina material.

When the tobacco material comprises lamina tobacco material, the lamina may be in whole leaf form. In some embodiments, the tobacco material comprises cured whole leaf tobacco. In some embodiments, the tobacco material substantially comprises cured whole leaf tobacco. In some embodiments, the tobacco material consists essentially of cured whole leaf tobacco.

In some embodiments, the tobacco material comprises stem tobacco material. The tobacco may comprise up to a 30 percent of stem material.

The process of “curing” green tobacco depends on the type of tobacco harvested. For example, Virginia flue (bright) tobacco is typically flue-cured, whereas Burley and certain dark strains are usually air-cured. The flue-curing of tobacco typically takes place over a period of five to seven days compared to one to two months for air-curing. Many major chemical and biochemical changes begin during the curing process and continue through the early phases of leaf drying. The conversion of the tobacco from its yellow to brown colour generally results in formation and substantial accumulation of nitrosamines, and an increased microbial content.

Different types of curing are used for different types of tobacco.

Virginia tobacco is generally 'flue-cured.' The tobacco leaves are hung in curing barns, where heated air is generated to dry the leaves. As the leaves lose moisture, they develop their distinct aroma, texture, and colour. The farmer must carefully guide this process, which takes up to a week, during which time the temperature of the heated air must be constantly monitored and gradually increased. Too much or too little heat at any stage of the process will have a negative impact on the quality of the tobacco.

Burley and oriental tobaccos are cured differently. Burley is 'air-cured' in barns where the heat and humidity come from natural ventilation. The curing process takes up to two months. Oriental tobacco is 'sun-cured' by hanging the leaves outdoors in the sun for about two weeks.

In the present text, the verbs “comprise” and “include” are synonyms and they both indicate a non-exhaustive list of features. The verb “consist” indicates an exhaustive list.

The invention is defined in the claims. However, below there is provided a non-exhaustive list of non-limiting examples. Any one or more of the features of these examples may be combined with any one or more features of another example, embodiment, or aspect described herein.

The invention could be inter alia defined by the following Examples.

Ex1. Method for treating tobacco material, the method comprising:

- providing a tobacco material
- fermenting the tobacco material to obtain fermented tobacco material, including:
- incubating the tobacco material under anaerobic conditions;
- and stopping the fermenting step when at least one of the following conditions is satisfied:
- the content of Lactic Acid is more than 10 times, preferably more than 20 times, more preferably more than 50 times, more preferably more than 70 times, preferably more than 80 times an initial amount of Lactic Acid in the tobacco material,
- the content of Reducing Sugars is lower than 0.5, preferably lower than 0.4, more preferably below 0.2, more preferably below 0.1 an initial amount or Reducing Sugars in the tobacco material,
- the content of Indole-3 Lactic Acid is more than 5 times, preferably more than 10 times, preferably more than 20 times, an initial amount of Indole-3 Lactic Acid in the tobacco material,
- the content of caffeic acid is more than 4 times, preferably more than 10 times, an initial amount of caffeic acid in the tobacco material,
- the content of quinic acid is more than 2 times, preferably more than 4 times, an initial amount of quinic acid in the tobacco material,
- the content of asparagine is lower than 0.5, preferably lower than 0.4, preferably lower than 0.3 an initial amount or asparagine, in the tobacco material
- the content of Glutamine is lower than 0.5, preferably lower than 0.4 an initial amount or Glutamine in the tobacco material,
- the content of L-Ornithine is more than 10 times, preferably more than 50 times, preferably more than 100 times an initial amount of L-Ornithine in the tobacco material,
- the content of L-Leucine is more than 2 times, preferably more than 4 times, an initial amount of L-Leucine in the tobacco material,
- the content of L-Lysine is more than 2 times, preferably more than 6 times, an initial amount of L-Lysine in the tobacco material,
- the fermentation index is more than 50, preferably more than 100, more preferably more than 250, more preferably more 400, wherein the fermentation index is obtained dividing the ratio between the content of Lactic Acid and an initial content of Lactic Acid in the tobacco material by the ratio between the content of Reducing Sugars and an initial content of Reducing Sugars in the tobacco material.

Ex2. Method according to Example 1 and further comprises an initial measuring step for measuring the initial content of at least one Lactic Acid, or Reducing Sugars, or Indole-3 Lactic Acid,

or quinic acid, or caffeic acid, or L-Ornithine, or Asparagine, or Glutamine, or L-Leucine, or L-Lysine, or fermentation index in the tobacco material.

Ex3. Method according to Example 1 or 2 and further comprising a measuring step for measuring the content of at least one Lactic Acid, or Reducing Sugars, or Indole-3 Lactic Acid, or quinic acid, or caffeic acid, or L-Ornithine, or Asparagine, or Glutamine or L-Leucine, or L-Lysine, or fermentation index in the tobacco material during the fermenting step.

Ex4. Method according to any one of Ex. 1 to 3, wherein if the tobacco material contains dark tobacco, it is provided for stopping the fermentation when at least one of the following conditions is satisfied: the content of 2,3 butanediol is more than 5 times, preferably more than 10 times an initial amount of 2,3 butanediol in the tobacco material or the content of diacetyl is more than 5 times, preferably more than 10 times an initial amount of diacetyl in the tobacco material.

Ex5. Method according to Example 4 and further comprising an initial measurement step for measuring the initial content of 2,3 butanediol or diacetyl in the tobacco material before the fermentation so as to obtain an initial amount respectively of 2,3 butanediol or diacetyl in the tobacco material.

Ex6. Method according to any of Examples 4 to 5 and further comprising a measuring step for measuring the content of 2,3 butanediol, or diacetyl in the tobacco material during the fermenting step.

Ex7. Method according to any one of Examples 1-6 and comprising during the fermenting step applying a pressure to the tobacco material comprised between 1000 kilograms per square meter and 15000 kilograms per square meter, preferably between 3000 kilograms per square meter and 12000 kilograms per square meter, more preferably between 5000 kilograms per square meter and 10000 kilograms per square meter.

Ex8. Method according to any one of Examples 1-7 and comprising during the fermenting step keeping the moisture content of the tobacco material during fermentation comprised between 10 percent in weight and 50 percent in weight preferably between 35 percent in weight and 45 percent in weight more preferably about 40 percent in weight of the total weight of the tobacco material.

Ex9. Method according to any one of Examples 1-8, wherein it is provided for continuing the fermenting step for a fermentation time of at least 1 month, preferably at least 2 months, more preferably at least 4 months, more preferably at least 6 months, even more preferably at least 8 months, preferably at least 10 months, more preferably at least 12 months.

Ex10. Method according to any one of Examples 1-9, wherein it is provided for continuing the fermenting step for a fermentation time of at least 24 months.

Ex11. Method according to any one of Examples 1-10 and further comprising drying the tobacco material to obtain a dried tobacco material having a moisture content comprised between 5 percent and 10 percent in weight of the total weight of the tobacco material.

Ex12. Method according to any one of claims 1-11, comprising a curing step for curing the tobacco material before the fermenting step.

Ex13. Method according to any one of Examples 1-12, comprising keeping the temperature of the tobacco material comprised between 21 degrees Celsius and 35 degrees Celsius, preferably between 25 degrees Celsius and 31 degrees Celsius.

Ex14. Method according to one or more of the preceding Examples, comprising the step of turning the tobacco material, preferably turning the tobacco material at a time interval of about 15 days, more preferably at a time interval of about 30 days, preferably between about 30 days and about 60 days.

Ex15. Method according to one or more of the preceding Examples, and comprising securing the tobacco material within a moisture retaining material.

Ex16. Method according to one or more of the preceding Examples, and comprising wetting the tobacco material in water before fermenting, so that a moisture content of the tobacco material comprised between about 10 percent in weight and about 50 percent in weight of the total weight, preferably between about 35 percent in weight and about 45 percent in weight of the total weight, preferably about 40 percent in weight of the total weight of the tobacco material is achieved.

Ex17. Method according to any one of the preceding Examples, and further comprising mixing a certain quantity of non-fermented tobacco material with a desired quantity of fermented tobacco material to obtain a tobacco material and then subjecting the tobacco material to the fermenting step, wherein the quantity of the fermented tobacco material in the tobacco material is comprised between 5 percent in weight and 10 percent in weight of the tobacco material.

Ex18. Method according to any of Examples 1 to 17 and further comprises a final measuring step for measuring the content of at least one of Lactic Acid, or Reducing Sugars, or Indole-3 Lactic Acid, or quinic acid, or caffeic acid, or L-Ornithine, or Asparagine, or Glutamine, or L-Leucine, or L-Lysine, or fermentation index in the tobacco material after the fermenting step.

Ex19. Method according to any one of Examples 1 to 18 and further provides for maintaining the pH of the tobacco material during fermentation comprised between 4.5 and 5.5, preferably about 4.8 and 5.4.

Ex20. Method for treating tobacco material, the method comprising:

- providing a tobacco material
- fermenting the tobacco material to obtain fermented tobacco material, the fermenting step including:

- incubating the tobacco material under anaerobic conditions, wherein during the fermenting step it is provided for applying a pressure to the tobacco material comprised between 1000 kilograms per square meter and 15000 kilograms per square meter, preferably between 3000 kilograms per square meter and 12000 kilograms per square meter, more preferably between 5000 kilograms per square meter and 10000 kilograms per square meter.

Ex21. Method for treating tobacco material, the method comprising:

- providing a tobacco material
- fermenting the tobacco material to obtain fermented tobacco material, the fermenting step including:
- incubating the tobacco material under anaerobic conditions, wherein it is provided for continuing the fermenting step for a fermentation time of at least 1 month, preferably at least 2 months, more preferably at least 4 months, more preferably at least 6 months, even more preferably at least 8 months, preferably at least 10 months, more preferably at least 12 months.

Ex22. Method for treating tobacco material, the method comprising:

- providing a tobacco material
- fermenting the tobacco material to obtain fermented tobacco material, the fermenting step including:
- incubating the tobacco material under anaerobic conditions, wherein during the fermenting step it is provided for keeping the temperature of the tobacco material comprised between 21 degrees Celsius and 35 degrees Celsius, preferably between 25 degrees Celsius and 31 degrees Celsius.

Ex23. Method according to any one of the preceding examples and comprising providing a database containing the amount of at least one of Lactic Acid, or Reducing Sugars, or Indole-3 Lactic Acid, or quinic acid, or caffeic acid, or L-Ornithine, or Asparagine, or Glutamine, or L-Leucine, or L-Lysine, or Butylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione, or Secoisolariciresinol (SECO), or fermentation index, or butanediol, or diacetyl in the fermented and/or partly fermented tobacco material.

Ex24. Method according to any one of the preceding examples and comprising providing a database containing the amount of at least one of Lactic Acid, or Reducing Sugars, or Indole-3 Lactic Acid, or quinic acid, or caffeic acid, or L-Ornithine, or Asparagine, or Glutamine, or L-Leucine, or L-Lysine, or Butylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione, or Secoisolariciresinol (SECO), or fermentation index, or butanediol, or diacetyl in the fermented and/or partly fermented tobacco material for a plurality of different tobacco materials.

Ex25. Method according to Examples 26 or 27 and comprising retrieving from at least one database the amount of at least one of Lactic Acid, or Reducing Sugars, or Indole-3 Lactic Acid, or quinic acid, or caffeic acid, or L-Ornithine, or Asparagine, or Glutamine, or L-Leucine, or L-Lysine, or Butylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione, or Secoisolariciresinol (SECO), or fermentation index, or butanediol, or diacetyl and comparing the retrieved value with the corresponding measured value in the tobacco material under fermentation.

Ex26. Method according to example 28 and further comprising continuing the fermenting step if the measured value of Lactic Acid, or Indole-3 Lactic Acid, or quinic acid, or caffeic acid, or L-Ornithine, or L-Leucine, or L-Lysine, or Butylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione, or Secoisolariciresinol (SECO), or fermentation index, or butanediol, or diacetyl is lower than the corresponding retrieved value and/or the value of Reducing Sugars, or Asparagine, or Glutamine, or fermentation index is higher than the corresponding retrieved value

Ex27. Tobacco material obtained according to a method of any one of Examples 1-26.

Ex28. Tobacco material obtained by a method comprising fermenting the tobacco material, the method including incubating the tobacco material under anaerobic conditions wherein at least one of the following conditions is satisfied in the tobacco material:

- the content of Lactic Acid is more than 10 times, preferably more than 20 times, more preferably more than 50 times, more preferably more than 70 times, preferably more than 80 times an initial amount of Lactic Acid in the tobacco ;
- the content of Reducing Sugars is lower than 0.5, preferably lower than 0.4, more preferably below 0.2, more preferably below 0.1 an initial amount or Reducing Sugars in the tobacco material,
- the content of Indole-3 Lactic Acid is more than 5 times, preferably more than 10 times, preferably more than 20 times, an initial amount of Indole-3 Lactic Acid in the tobacco material,
- the content of caffeic acid is more than 4 times, preferably more than 10 times, an initial amount of caffeic acid in the tobacco material,
- the content of quinic acid is more than 2 times, preferably more than 4 times, an initial amount of quinic acid in the tobacco material,
- the content of asparagine is lower than 0.5, preferably lower than 0.4, preferably lower than 0.3 an initial amount or asparagine in the tobacco material,
- the content of Glutamine is lower than 0.5, preferably lower than 0.4 an initial amount or Glutamine in the tobacco material,
- the content of L-Ornithine is more than 10 times, preferably more than 50 times, preferably more than 100 times an initial amount of L-Ornithine in the tobacco material,

- the content of L-Leucine is more than 2 times, preferably more than 4 times, an initial amount of L-Leucine in the tobacco material,
- the content of L-Lysine is more than 2 times, preferably more than 6 times, an initial amount of L-Lysine in the tobacco material,
- the fermentation index is more than 50, preferably more than 100, more preferably more than 250, more preferably more 400, wherein the fermentation index is obtained dividing the ratio between the content of Lactic Acid in the tobacco material and the content of Lactic Acid in the non-fermented tobacco material by the ratio between the content of Reducing Sugars in the tobacco material and the content of Reducing Sugars in the non-fermented tobacco material.

Ex29. Tobacco material containing at least one of the following features:

- Lactic Acid in an amount that is more than 10 times, preferably more than 20 times, more preferably more than 50 times, more preferably more than 70 times, more preferably more than 80 times, an initial amount of Lactic Acid in the tobacco material.
- Reducing Sugars in an amount that is lower than 0.5, preferably lower than 0.4, more preferably below 0.2, more preferably below 0.1 an initial amount or Reducing Sugars in the tobacco material,
- Indole-3 Lactic Acid in an amount that is more than 5 times, preferably more than 10 times, preferably more than 20 times, an initial amount of Indole-3 Lactic Acid in the tobacco material.
- caffeic acid in an amount that is more than 4 times, preferably more than 10 times, an initial amount of caffeic acid in the tobacco material,
- quinic acid in an amount that is more than 2 times, preferably more than 4 times, an initial amount of quinic acid in the tobacco material,
- asparagine in an amount that is lower than 0.5, preferably lower than 0.4, preferably lower than 0.3 an initial amount or asparagine in the tobacco material,
- Glutamine in an amount that is lower than 0.5, preferably lower than 0.4 an initial amount or Glutamine in the tobacco material,
- L-Ornithine is more than 10 times, preferably more than 50 times, preferably more than 100 times an initial amount of L-Ornithine in the tobacco material,
- L-Leucine in an amount that is more than 2 times, preferably more than 4 times, an initial amount of L-Leucine,
- L-Lysine in an amount that is more than 2 times, preferably more than 6 times, an initial amount of L-Lysine,

- a fermentation index is more than 50, preferably more than 100, more preferably more than 250, more preferably more 400, wherein the fermentation index is obtained dividing the ratio between the content of Lactic Acid in the tobacco material and the content of Lactic Acid in the non-fermented tobacco material by the ratio between the content of Reducing Sugars in the tobacco material and the content of Reducing Sugars in the non-fermented tobacco material.

Ex30. Tobacco material according to the preceding Example, wherein said tobacco material is obtained by a process comprising fermenting the tobacco material to obtain treated tobacco material, including: incubating the tobacco material under anaerobic conditions.

Ex31. Tobacco material containing at least 20 milligrams per gram, preferably at least 50 mg per gram, more preferably at least 60 milligrams per gram of Lactic Acid in total dry weight basis.

Ex32. Tobacco material comprising less than 3 percent of total Reducing Sugars in total dry weight basis.

Ex33. Tobacco material comprising less than 300 milligrams per kilogram of asparagine in total dry weight basis.

Ex34. Tobacco material comprising less than 70 milligrams per kilogram of glutamine in total dry weight basis.

Ex35. Tobacco material comprising more than 10000 milligrams per kilogram in total dry weight basis of total free amino acids.

Ex36. Tobacco material comprising at least 1 microgram per gram, preferably at least 2 micrograms per gram, more preferably at least 2.5 micrograms per gram of Indole-3 Lactic Acid.

Ex37. Tobacco material according to one or more of Examples from 27 to 36, wherein the tobacco material is cured.

Ex38. Tobacco material according to one or more of Examples from 27 to 37, wherein the tobacco material is grinded.

Ex39. Aerosol generating article comprising a tobacco material containing between about 2.5 percent by weight in total dry weight basis and 100 percent by weight in total dry weight basis , preferably at least about 4 percent by weight in total dry weight basis, preferably at least about 10 percent by weight in total dry weight basis, preferably at least about 20 percent by weight in total dry weight basis of a the tobacco material according to any one of examples 27 to 38.

Ex40. Aerosol generating article comprising a tobacco material containing at least 5 milligrams per gram, preferably 10 milligrams per gram of Lactic Acid.

Ex41. Aerosol generating article comprising a tobacco material containing less than 300 milligrams per kilogram of asparagine in total dry weight basis.

Ex42. Aerosol generating article comprising a tobacco material containing than 70 milligrams per kilogram of glutamine in total dry weight basis.

Ex43. Aerosol generating article comprising a tobacco material containing more than 10000 milligrams per kilogram of asparagine in total dry weight of total free amino acids.

Examples will now be further described with reference to the figures in which:

- FIG 1 and FIG. 2 are histograms representing the quantity of Lactic Acid in the tobacco material of Example 1 and Example 2, respectively, measured before (0T) and after 6 months (3T) of fermentation according to the invention;
- FIG. 3 and FIG. 4 are histograms representing the quantity of Total alkaloid (TA) levels (percent in total dry weight basis DW) in the tobacco material of Example 1 and Example 2, respectively, measured before (0T) and during fermentation according to the invention;
- FIG. 5 and FIG. 6 are histograms representing the quantity of glutamine and glutamic acid respectively, in the tobacco material (in total dry weight basis, DW) of Example 1 and Example 2, measured before (0T) and during fermentation according to the invention;
- FIG. 7 and FIG. 8 are histograms representing the quantity of asparagine and aspartic acid respectively, in the tobacco material (in total dry weight basis, DW) of Example 1 and Example 2 measured before (0T) and during fermentation according to the invention;
- FIG. 9 and FIG. 10 are histograms representing respectively the quantity of total alkaloids (FIG. 9) and Reducing Sugars (FIG. 10) in the tobacco material (in total dry weight basis, DW) of Example 3, respectively, measured before (VG-BF), during fermentation and after fermentation (VG-AF) according to the invention.
- FIG. 11 shows the TA (Total Alkaloids), RS (Reducing Sugars) and ammonia (NH3) in non-fermented Virginia (VG) material (SM) during the fermentation process (T1 toT7) and after fermentation (AF), in percent in total dry weight basis, DW.
- FIG. 12 shows the amount of Glucose, Fructose, citrate, Malate, Pyruvate and Lactate during the anaerobic fermentation of Virginia tobacco in non-fermented Virginia material (SM) and after fermentation (AF).
- FIG. 13A and 13B shows consumed (FIG. 13A) and produced (FIG. 13B) free amino acids before and after fermentation of Virginia tobacco. Ratio data expressed from metabolomic analyses (n=3), statistics are paired t-test (* $p<0.05$;** $p<0.01$; *** $p<0.001$);
- FIG. 14 shows the production of quinic and caffeic acids in the fermentation of Virginia tobacco. Ratio data expressed from metabolomic analyses (n=3), statistics are paired t-test (* $p<0.05$;** $p<0.01$; *** $p<0.001$);

- FIG. 15 shows the accumulation of Indole-3-Lactic Acid in fermented tobacco (AF) compared to non-fermented Virginia tobacco (SM) from enzymatic L-Tryptophan degradation. Ratio data expressed from metabolomic analyses (n=3), statistics are paired t-test (* $p<0.05$;** $p<0.01$; *** $p<0.001$)
- FIG. 16 shows the percentage of TA, RS, NO3 and NH3 of Virginia tobacco material of Example 4 at start (0 month) and after 3 and 6 months fermentation. Analyses performed by scalar method.
- Fig. 17 shows the change of Indole-3-Lactic Acid in the tobacco material of Example 1 (RAJ) and of Example 2 (HS) between the non-fermented material (control) and the fermented material (HF).
- Fig. 17A shows the change of Indole-3-Lactic Acid in the tobacco material of Example 4 (VG-CH) in the non-fermented tobacco material (NF) and in fermented tobacco material (F).
- Fig. 18 shows the change of L-Ornithine in the tobacco material of Example 1 (RAJ) and of Example 2 (HS) between the non-fermented material (control) and the fermented material (HF);
- Fig. 19 shows the change of BHHPPD in the tobacco material of Example 1 (RAJ) and of Example 2 (HS) between the non-fermented material (control) and the fermented material (HF);
- Fig. 20 shows the change of Secoisolariciresinol in the tobacco material of Example 1 (RAJ) and of Example 2 (HS) between the non-fermented material (control) and the fermented material (HF);

A first and a second tobacco material of the same tobacco type but having a different processing before fermentation have been prepared. The tobacco material is Kasturi tobacco.

Example 1

Dark tobacco leaf material has been fully sun-cured for about 10 days. The sun cured leaves have been stripped to keep only the lamina (hand stripped leaves). This tobacco material is referred to as “HS”.

The tobacco material was conditioned to obtain a moisture of circa 30 percent. Samples of this tobacco material conditioned but not fermented yet are called 0T (“starting material”).

The conditioned tobacco material is then introduced in three barrels, in each barrel circa 100 kilograms of tobacco material is present. Before the introduction, the tobacco material is wrapped in a material maintaining the acquired moisture.

Pressure is applied to each barrel. The pressure is comprised between between 1000 kilograms per square meter and 4000 kilograms per square meter.

After 1 month (sample called 1T), 2.5 months (sample called 2T), 6 months (sample called 3T) and 8.5 months (sample called 4T), the barrels were opened, and the samples collected at least in triplicate in each barrel before tobacco turning and readjustment of the moisture content to approximatively 30 percent ± 5 percent.

During the heavy fermentation process under fully anaerobic conditions, the temperature inside the barrels did not particularly increase (it remained within the following temperature range: between 27 degrees Celsius and 31 degrees Celsius). The fermentation has been stopped after 8.5 months.

Example 2

Dark tobacco leaf material has been yellowed for two days and rapidly chopped in cut-filler. This tobacco material contains both lamina and ribs. The chopped leaves containing both lamina and mid- ribs were sun-dried for two days. Samples of this tobacco material are named in the following "CC".

The tobacco material was conditioned to obtain a moisture content of circa 30 percent. Samples of this tobacco material conditioned but not fermented yet are called 0T ("starting material").

The conditioned tobacco material is then introduced in three barrels, in each barrel circa 100 kilograms of tobacco material is present. Before the introduction, the tobacco material is wrapped in a material maintaining the acquired moisture.

Pressure is applied to each barrel. The pressure is comprised between between 1000 kilograms over square meter and 4000 kilograms over square meter.

After 1 month (sample called 1T), 2.5 months (sample called 2T), 6 months (sample called 3T) and 8.5 months (sample called 4T), the barrels were opened, and the samples collected at least in triplicate in each barrel before tobacco turning and readjustment of the moisture content to approximatively 30 percent ± 5 percent.

During the heavy fermentation process under fully anaerobic conditions, the temperature inside the barrels did not particularly increase (it remained within the following temperature range: between 27 degrees Celsius and 31 degrees Celsius). The fermentation has been stopped after 8.5 months.

Visual observations

The initial tobacco material was changing already after 2.5 month (samples 2T) of fermentation, the color of both HS and CC leaves became darker, the tobacco smell expressing nice caramel-buttery and fermented complex notes. The dark color was more marked in the fermented HS leaves compared to the CC leaves at the end of the process (8.5 month, 4T), likely due to the presence of leaf mid-rib in the CC leaves.

Chemical analysis

In the following, when a value relative to a sample is mentioned, the given value represents an average of several values obtained for each sample of the same type.

The pH of the samples of tobacco material, both CC and HS, became acidic reaching 3.2, after fermentation conditions have been applied for 2.5 months (as found in sample 2T). This reflects the process of anaerobic fermentation involving sugars degradation, which usually produces organic acids like (acetic and/or) Lactic Acids. The starting pH of the tobacco material is generally comprised between 5 pH and 6 pH.

Figs. 1 and 2 show the presence of Lactic Acid in the tobacco material. As shown by the figures (figures 1 represent Lactic Acid content in HS leaves and figure 2 in CC leaves), before fermentation, there is absence of Lactic Acid in all samples (three samples 0T per tobacco material – CC or HS – are shown). After fermentation (in this case after 6 months, three samples for tobacco material called 3T, shown for both tobacco materials – CC or HS), all samples, both CC and HS leaves, show the presence of Lactic Acid, albeit in variable amount.

Alkaloids were not or only slightly degraded during the fermentation. The total alkaloids (TA) content in percent in total dry weight basis (indicated as percent DW in the figures) is shown in Figure 3 (HS leaves) and Figure 4 (Chopped leaves, CC leaves). The content of total alkaloids remained quite stable during the fermentation. After 8.5 month (4T), only 4 percent were degraded in HS and 9 percent in CC leaves. Although statistically relevant, such small variation may just result from sampling. Some limited alkaloid hydrolase activities may not be excluded. Total alkaloids were analyzed in samples collected during the heavy fermentation process at start (0T, n=6 samples have been analyzed), after 1(1T, n=9), 2.5(2T, n=9), 6 (3T, n=9) and 8.5 (4T, n=12) months. T-tests (test statistics) were performed for comparison with the control, unfermented cured tobacco (0T). The results are shown in Figures 3 and 4 indicating the p-value, where the p-value is shown as follows:

*, $p<0.05$;

**, $p<0.01$ and

***, $p<0.001$.

Sample 4T of HS leaves and sample 3T of CC leaves have a p-value < 0.01 and Samples 1T and 4T of CC leaves have a p-value < 0.001. This indicates a statistical significant difference between the fermented tobacco material and the non-fermented one.

The nitrate content was not affected by the heavy fermentation process. However, some impact was observed on tobacco specific nitrosamines (TSNA): NNN (N'-nitrosonornicotine), NNK (nicotine-derived nitrosamine ketone) and NAT (N'-nitrosoanatabine). No changes were measured on NNK and NAT after 8.5 months fermentation. However, an increase of NNN was observed in both HS (3x increase) and CC (5-6x increase). As nornicotine, the precursor of NNN before nitrosation did not increase correspondingly, therefore NAT and NNK, but not NNN, may be partially degraded by bacteria during the fermentation run,

since NNK and NAT first increased by a factor 2 till 2.5 months fermentation and then decreased to reach the initial value of non-fermented tobacco. This observation may mean that nitrosation of alkaloids occurs during heavy fermentation.

The evolution of sugars and free amino acids during the heavy fermentation according to the invention has been analyzed. The measurements performed in the samples of tobacco material are collected in Table 1. Table 1 shows the evolution of sugars and amino acids during the heavy fermentation process from the untreated tobacco material sample (samples 0T) to 8.5 months of fermentation process (samples 4T) under fermentation conditions in barrels containing either hand-stripped (HS) or Chopped (CC) leaves, as in Example 1 and Example 2. All values in the table are in total dry weight basis. The units of Reducing Sugars are in percent in total dry weight basis, while the free amino acids are in milligram per kilogram of total dry tobacco material. A drop of Reducing Sugars appeared after 2.5 month (2T, see Table 1) in phase with the color change and the slurry acidification. Glucose and fructose are two tobacco leaf substrates that anaerobic bacteria may metabolize in the fermentation barrels. Conversely, most of the amino acids increased during the process. Both asparagine and glutamine strongly decreased. Altogether, these observations may indicate that the main fermentation activities occurred between the first and the third month. Proline was not degraded under anaerobic fermentation (see Table 1). Ornithine strongly increased during fermentation (> 100 times) in both HS and CC, as well as citrulline (data obtained from metabolomic analyses between 0T and 3T) increasing by a factor 16 in HS and 2 in CC. This may indicate that (plant-derived) Lactic Acid bacteria are active in the tobacco fermenting barrels, since such bacteria are described to produce ornithine and citrulline at high levels (Rakhimuzzaman *et al., Biol Pharm Bull.* 2019;42(9):1581-1589).

		HS-0T	HS-1T	HS-2T	HS-3T	HS-4T	CC-0T	CC-1T	CC-2T	CC-3T	CC-4T
Sugars (%DW)	GLUCOSE	3.0	3.2	0.4	0.3	0.5	4.0	4.6	0.7	0.9	1.0
	FRUCTOSE	4.3	4.1	0.3	0.2	0.2	4.9	5.1	0.4	0.3	0.3
	SUCROSE	0.1	0.0	0.0	0.0	0.0	0.9	0.0	0.0	0.0	0.0
	MALTOSE	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
	SUM OF SUGARS	**7.4**	**7.3**	**0.7**	**0.5**	**0.7**	**9.8**	**9.7**	**1.1**	**1.2**	**1.3**
Free Amino Acids (mg/kg DW)	ASPARTIC ACID	252.0	951.3	2459.0	2702.3	2933.2	160.7	1492.0	1742.7	2184.7	2074.0
	GLUTAMIC ACID	518.7	813.3	1293.7	1185.0	1219.8	787.0	949.7	1201.7	986.0	1002.0
	ALANINE	601.3	762.3	1231.3	1374.7	1524.3	293.0	624.7	1084.0	1403.7	1652.2
	ARGININE	41.3	123.7	147.0	180.0	210.3	17.0	237.0	140.3	267.3	226.8
	ASPARAGINE	**1604.3**	**1920.7**	**212.0**	**42.7**	**22.3**	**1747.0**	**640.0**	**287.7**	**124.3**	**139.0**
	PROLINE	4165.3	5108.7	4885.0	4838.3	4920.8	2962.3	3502.0	3017.7	3415.3	3417.0
	PHENYLALANINE	234.0	400.7	336.0	402.0	414.5	236.3	386.7	243.0	312.7	327.8
	GLYCINE	68.0	111.0	251.7	342.3	376.3	48.0	155.7	254.0	344.0	363.8
	GLUTAMINE	**1456.0**	**692.0**	**56.7**	**25.3**	**0.0**	**1921.0**	**314.3**	**57.7**	**17.0**	**52.5**
	ISOLEUCINE	31.7	99.0	165.3	217.3	182.0	43.0	197.7	188.0	258.7	215.3
	HYSTIDINE	122.7	185.0	142.0	136.0	115.5	160.3	145.7	109.7	109.7	91.0
	LEUCINE	113.3	238.3	372.7	464.7	621.5	143.7	473.7	334.3	419.7	585.8
	LYSINE	54.0	131.7	183.0	241.0	256.2	40.0	242.3	240.0	323.0	340.5
	METHIONINE	30.0	34.0	16.7	11.0	20.3	16.0	38.7	12.0	11.0	28.8
	ORNITHINE	0.0	0.0	92.7	113.0	87.8	18.0	0.0	125.0	97.0	89.2
	SERINE	297.3	393.3	453.7	495.7	535.3	277.3	444.7	500.7	583.7	609.7
	TYROSIN	81.3	135.3	221.3	274.3	247.3	111.0	233.3	246.0	318.7	277.8
	THREONINE	156.7	272.0	346.7	433.3	449.5	184.0	350.0	392.7	470.3	473.3
	VALINE	279.0	398.3	468.0	523.3	496.0	278.3	593.0	627.3	784.0	626.5
	GABA	326.0	465.7	507.0	468.3	438.0	358.7	980.7	842.3	860.7	845.7
	TOTAL FREE AMINO ACIDS	10459.7	13270.7	13886.3	14494.0	15225.2	9813.0	12027.7	11658.7	13293.0	13520.2

TABLE 1

In figures 5 – 8, the amount of glutamine and asparagine in the tobacco material is shown. As shown by Figures 5 - 8 and based on the data presented in Table 1, deamination of glutamine and asparagine occurring during the heavy fermentation process of both HS and CC leaves may be correlated with the concomitant increase of glutamate and aspartate, respectively. This suggests that fermenting bacteria produce specific glutaminase(s) and asparaginase(s) to assimilate C and N from amino acid resources. Both reactions produce ammonia that increased twofold during the anaerobic fermentation process of both HS and CC leaves. Figures 5 and 6 show the level of glutamine (white histograms) and glutamic acid (black histograms) in HS leaves and CC leaves, respectively. It is clear from the figures that during fermentation glutamine decreases and glutamic acid increases. Figures 7 and 8 show the level of asparagine (striped histograms) and aspartic acid (black histograms) in HS leaves and CC leaves, respectively. It is clear from the figures that during fermentation asparagine decreases and aspartic acid increases.

A metabolomic study was performed to identify marker molecules or pathways related to the tobacco leaf anaerobic fermentation process. Sugar resources such as glucose and fructose present in the starting material (control) of both HS and CC leaves may be used as a source of energy by the anaerobic bacteria (see Table 1). In the absence of oxygen, the glycolysis pathway transforms glucose (or fructose) into pyruvate producing 2 ATP and 2 NADH+H+. Other organic and rich carbon compounds that may be rapidly used by anaerobic bacteria are citrate and malate (Bintsis, T, 2018, *AIMS Microbiology*, 4(4): 665–684), both being the most abundant organic acids in plants. Citrate and malate, like Reducing Sugars, are also metabolized during the tobacco heavy fermentation: it is shown from chemical analysis of the samples that more than 60percent of the glucose and fructose, citrate and malate present in the starting tobacco material (samples 0T), hand-stripped and chopped leaves are catabolized after 6 months of heavy fermentation (samples 3T). Another observation that can be coupled to the consumption of such organic molecules is the increase of pyruvate (13-14 times) in both HS and CC fermented tobacco material. Pyruvate is the substrate of several reactions that may occur under anaerobic conditions: (1) the production of D-lactate, mostly to regenerate NAD+ for the glycolytic reaction; (2) the production of acetate, diacetyl and 2,3 butanediol that may contribute to the delivery of aromatic compounds and flavours in heavy fermented tobacco. Pyruvate may lead to the generation of aromatic compounds, like 2,3-butanediol or lactate as a product of Lactic Acid bacteria.

Two other pathways emerged from the metabolomic analyses of heavy fermented tobacco: (1) the degradation of tryptophan and (2) the catabolism of chlorogenic acid.

Regarding tryptophan degradation, the pathway has been described by Ummadi and Weimer (2001, J. Dairy Sci. 84:1773–1782) for cheese bacteria and adapted accordingly. In this case, more than 78percent of the tryptophan present in the starting tobacco material (samples 0T) is catabolized after 6 months of fermentation (samples 3T) in both HS and CC leaves. The pathway indicated that the product resulting from

such a catabolic reaction is principally indole-3-Lactic Acid. This is illustrated by an increase of 14 times and 28 times in HS and CC leaves, respectively. No other compound belonging to this pathway showed such an increase. No specific aromatic properties had been reported for this compound.

Chlorogenic acid (CGA), an important biologically active dietary polyphenol, is produced by certain plant species, like tobacco, and is a major component of coffee. In heavy fermented tobacco leaf, CGA is completely degraded after the anaerobic fermentation process. On the other side, products resulting from the catabolism of CGA, namely quinic and caffeic acids, increased in both HS and CC leaves after 6 months fermentation. This likely results from bacterial cinnamoyl esterase activities as documented by Guglielmetti et al. (2008, *Applied and Environmental Microbiology*, 74, 4: 1284–1288). Therefore, part of the quinic and caffeic acid pools likely result from the hydrolysis of CGA, whereas none of them was reported to have flavor properties.

The presence of elevated pyruvate, indole-3-Lactic Acid and the lack of chlorogenic acid in heavy fermented tobacco compared to cured tobacco can make them useful as chemical markers.

For the purpose of the present description and of the appended claims, except where otherwise indicated, all numbers expressing amounts, quantities, percentages, and so forth, are to be understood as being modified in all instances by the term "about". Also, all ranges include the maximum and minimum points disclosed and include any intermediate ranges therein, which may or may not be specifically enumerated herein. In this context, therefore, a number A is understood as A ± 10 percent of A. Within this context, a number A may be considered to include numerical values that are within general standard error for the measurement of the property that the number A represents. The number A, in some instances as used in the appended claims, may deviate by the percentages enumerated above provided that the amount by which A deviates does not materially affect the basic and novel characteristic(s) of the claimed invention. Also, all ranges include the maximum and minimum points disclosed and include any intermediate ranges therein, which may or may not be specifically enumerated herein.

The tobacco material of Example 1 and 2 have also been tested for some additional chemical compounds which could be connected with the fermentation.

Fig. 17 shows the change of Indole-3-Lactic Acid in the tobacco material of example 1 (RAJ) and of example 2 (HS) between the non-fermented material (control) and the fermented material (HF). These data show an increase of the content of Indole-3-Lactic Acid in the fermented material.

Fig. 18 shows the change of L-Ornithine in the tobacco material of example 1 (RAJ) and of example 2 (HS) between the non-fermented material (control) and the fermented material (HF), indicated as quantity of L-

Ornithine, and also as absolute change. These data show an increase of the content of L-Ornithine in the fermented material.

Fig. 19 shows the change of BHHPPD in the tobacco material of example 1 (RAJ) and of example 2 (HS) between the non-fermented material (control) and the fermented material (HF). These data show an increase of the content of BHHPPD in the fermented material.

Fig. 20 shows the change of Secoisolariciresinol in the tobacco material of example 1 (RAJ) and of example 2 (HS) between the non-fermented material (control) and the fermented material (HF). These data show an increase of the content of Secoisolariciresinol in the fermented material.

Example 3

A further trial has been conducted in Asia on sample of Virginia tobacco. Tobacco leaf material has been flue-cured as a standard procedure.

The tobacco cured material, in form of strips, was then conditioned to obtain moisture of about 30 percent. Samples of this tobacco material conditioned but not fermented yet are called SM (starting material before fermentation).

The conditioned tobacco material is then introduced in two barrels, in each barrel circa 100 kilograms of tobacco material is present. Before the introduction, the tobacco material is wrapped in a material maintaining the acquired moisture. The two barrels containing Virginia tobacco material were subjected to anaerobic fermentation as described in Example 1. The temperature inside the fermentation barrel and the pH of the tobacco material under fermentation were monitored during the whole experiment.

After 1 month (sample called 1T), 2 months (sample called 2T), 3 months (sample called 3T), 4 months (sampled called 4T), 5 months (sampled called 5T), 6 months (sample called 6T), 7 months (sample called 7T) and 8 months (sample called AF, after fermentation), the barrels were opened.

The tobacco material in the two barrels has been turned monthly during the 7 months of the experiment.

Samples have been collected before fermentation (VG-SM: starting material, 6 replicates), during the fermentation process (in all months from VG-T1 to VG-T7, 3 replicates per barrel) and after fermentation (VG-AF: after fermentation, 6 replicates), as indicated in Table 2 reported below. The features of the sample have been analyzed.

Tobacco type	SM	T1	T2	T3	T4	T5	T6	T7	AF
VG-01	07.11.2019	10.12.2019	08.01.2020	07.02.2020	10.03.2020	06.04.2020	06.05.2020	10.06.2020	10.07.2020
VG-02	07.11.2019	11.12.2019	08.01.2020	10.02.2020	10.03.2020	06.04.2020	06.05.2020	10.06.2020	10.07.2020

Table 2

During the sample collection, the tobacco material has been turned and the moisture content of the tobacco material has been readjusted to approximatively 30 percent ± 5 percent.

During the heavy fermentation process under fully anaerobic conditions, no major changes of temperature were observed during the fermentation process moving linearly from 30 degrees Celsius at the beginning of the fermentation (VG-T1) to 26 degrees Celsius at the end of the fermentation (VG-AF). The temperature has been measured inside the barrels using captors.

The pH of the tobacco material did not change significantly during the fermentation run (T1 to AF) staying at 5.1 ± 0.3.

Visual observations

As seen for the Kasturi tobacco material, the color of the tobacco material at the end of the fermentation process (VG-AF) became remarkably darker compared to the starting material (VG-SM). However, after 4 month of anaerobic fermentation (VG-T4), and thus after 4 turnings, the Virginia tobacco material did not show a so dark coloration indicating possibly that 4 months are not sufficient to get full fermentation of Virginia tobacco material at the experimental condition. After 8 months of anaerobic fermentation (VG-AF), and thus after 8 turnings, the Virginia tobacco material has a darker color than after 4 months, thus indicating that a full fermentation of Virginia tobacco material took place. Moreover, nice scented and floral odors were perceived after 8 months.

Chemical analysis

In the following, when a value relative to a sample is mentioned, the given value represents an average of several values obtained for each sample of the same type, replicate.

Figure 11 shows the evolution of total alkaloids (TA) during the fermentation process, Reducing Sugars (RS) and ammonia (NH3) during the fermentation process. These data confirm that alkaloids and in particular nicotine (not shown) are not impacted by anaerobic fermentation. The bacteria did not consume major alkaloids as fermenting substrates. No increase of ammonia were observed when using Virginia as fermented material, and the nitrate which was not present in this Virginia material did not shown any increase during and after the fermentation process. Fig. 11 shows the TA (Total Alkaloids), RS (Reducing Sugars) and ammonia (NH3) in non-fermented Virginia (VG) material (SM) during the fermentation process (T1 toT7) and after fermentation (AF). The data are expressed in percent DW. The data of TA (Total Alkaloids), RS (Reducing Sugars) and ammonia (NH3) in non-fermented Virginia (VG) material (SM) and after fermentation (AF) are also reported in the histograms of Figure 11, in which the difference of values between non fermented and fermented material is immediately evident.

On the other hand, as already observed in Example 1 and Example 2, Reducing Sugars were used as substrate by fermenting bacteria. Therefore about 60percent of the Reducing Sugars (RS) were oxidized during the 8 month of fermentation, moving from 18.3 percent (VG-SM) to 7.4 (VG-AF) percent in dry weight (DW). It is possible that a longer period of fermentation would lead to a higher percentage of degradation of Reducing Sugars.

Further chemical analyses have been performed to compare the compounds in the starting material (VG-SM) and in the fermented material (VG-AF).

The analyses show a strong significant increase (~10x) of lactate (HS ID VG-19-20-NF, HS ID VG-19-20-F in Table 3) in the fermented material (VG-AF) compared to non-fermented material (VG-SM). Moreover the amount of glucose and fructose in the fermented material (VG-AF) is considerably lower than the content of glucose and fructose in the starting material (VG-BF). Glucose in fermented material (VG-AF) is lower than 0.6 the glucose in non-fermented material (VG-SM), fructose in fermented material (VG-AF) is lower than 0.4 the fructose in non-fermented material (VG-SM). Thus about 60percent reduction after fermentation of Reducing Sugars can be observed. In addition, the organic acids, citrate and malate, are also impacted by the fermentation, as also shown in Figure 1 for Dark tobacco. Fig. 12 shows catabolism of tobacco Reducing Sugars, citrate and malate followed by an accumulation of pyruvate and Lactic Acid (Lactic Acid fermentation) during the anaerobic fermentation of Virginia tobacco. Ratio data expressed from metabolomic analyses (n=3), statistics are paired t-test (* $p<0.05$;** $p<0.01$; *** $p<0.001$)

Chemical analyses also confirmed that asparagine and glutamine, are on the side to Reducing Sugars also consumed by anaerobic bacteria, that other free amino acids, like glutamate, histidine, proline and tryptophan are also significantly degraded after fermentation of Virginia tobacco and on the contrary other aminoacids increased after fermentation of Virginia tobacco, particularly L-Leucine and L-lysine (see Figure 13A and 13B). Fig. 13A and 13B show respectively consumed and produced free amino acids before and after fermentation of Virginia tobacco. Ratio data expressed from metabolomic analyses (n=3), statistics are paired t-test (* $p<0.05$;** $p<0.01$; *** $p<0.001$)

Fig. 14 shows the change in the content of quinic acid and Caffeic acid. As already observed in Example 1 and Example 2, quinic and caffeic acid largely increased after the fermentation when the content of these compounds in the fermented tobacco material is compared with the content in the non-fermented tobacco material. This is probably as the result of cinnamoyl esterase catabolizing chlorogenic acid. Besides powerful antioxidant activity, increasing collagen production and prevention of premature aging, caffeic acid has demonstrated antimicrobial activity and may be promising in the treatment of dermal diseases. On the other side, Quinic acid is also a potent drug candidate to combat prostate cancer.

Indole-3-Lactic Acid (Figure 15), largely increased (>10x) during the tobacco fermentation process and originates from the catabolism of tryptophan. Therefore, as Lactic Acid, the organic acid indole-3-Lactic Acid is also a good marker for Lactic Acid fermentation.

Example 4

This experiment has been conducted in Switzerland, in the same experimental condition used in Example 3.

For this experiment have been used metal wine barrels able to press a wet tobacco substrate for a duration between two to ten months. A pressing equipment used for grapes and customized for the needs of the project has been used. The barrel has a flat bottom, custom disc diameters and custom pressure gauge. It can press between 0 and 8 kilograms per square centimeter. The pressing can be either manual or triggered by an electric motor. As an optional part, a wooden disc is attached to the stainless-steel pressing disc. The fermentation tank has been modified for the purpose of the test, having a height of 70 centimeters, a diameter of 57 centimeters, a capacity of 100 kilograms and an opening at the back of the metal tank (barrel) to help unpacking the substrate when turning the material in the middle or at the end of the fermentation run.

FC tobacco strips from Brazil (CX B) was used as fermenting substrate preconditioned with water to reach a final humidity of between about 30 percent by weight to about 50 percent by weight of water. About 100 kilogramsof tobacco is material is loaded in the fermentation tank and pressed. The pressure is kept between about 0.5 kilograms per square centimeter to about 1 kilogram per square centimeter. The tobacco material is maintained at a relative humidity of about 50 percent by weight and at a temperature of about 22 Celsius degrees during the whole fermentation process (6 months). After three months, the tobacco material is unloaded from the barrel, separated, mixed, turned. The tobacco material is then added with between about 30 percent by weight of water to about 50 percent by weight of water and then reloaded in the barrel.

Pressure and water levels were regularly monitored and corrected to prevent deviation from the targets and any aerobic fermentation. Temperature inside the tank did not change during the whole run, as observed previously.

After 6 months, the tobacco material was pre-dried on a belt-oven for a total drying time of about 8 minutes at different temperatures ranges. The tobacco material is firstly subjected to a temperature of about 40 Celsius degrees, then to a temperature of about (70 Celsius degrees and then to a temperature of about 60 Celsius degrees . The pre-drying process allows to obtain a tobacco material with about 25 percent by weight of Relative humidity OV [percent] to allow the cutting of the strips into finer particles. The tobacco material is then cut in particles of about 1 millimeters of cut-width. The tobacco material is then dried to its final moisture content of 10 percent by weight to about 15 percent by weight OV [percent]. The drying step took

place in a rotary dryer at a temperature comprised between about 90 Celsius degrees to about 100 Celsius degrees and a pressure of about 0.6 bar for a period of about during 10 minutes. The tobacco material is then finally grinded with a specification of 70 micrometers for short storage.

The tobacco material in the two barrels has been turned every two months during the 6 months of the experiment.

Samples have been collected before fermentation (VG-SM: starting material, 6 replicates), during the fermentation process after 3 months from the beginning of the fermentation process and after fermentation (VG-AF: after fermentation, 6 replicates).

Visual observations

The color of the tobacco material at the end of the fermentation process (VG-AF) became remarkably darker compared to the starting material (VG-SM). After 6 months of anaerobic fermentation the Virginia tobacco material shows a dark coloration indicating that a full fermentation of Virginia tobacco material has occurred.

Chemical analysis

In the following, when a value relative to a sample is mentioned, the given value represents an average of several values obtained for each sample of the same type, replicate.

Figure 16 shows the difference between the content of total alkaloids (TA) during the fermentation process, Reducing Sugars (RS), Nitrate (NO_3) and ammonia (NH_3) before fermentation and after three months of fermentation and at the end of the fermentation process. Total alkaloids (TA) are not impacted by the anaerobic fermentation process. Reducing Sugars are consumed by the anaerobic bacteria and their content in the tobacco material decrease with fermentation. At the end of the fermentation the level of Reducing Sugars in the tobacco material is very low. In this case Reducing Sugars were almost completely consumed after 6 month fermentation and one turning only. These data are comparable with the data obtained in example 1, 2 and 3.

Chemical analyses show that Lactic Acid is produced during heavy fermentation. According to the metabolic pathways present in Lactic Acid bacteria, Lactic Acid originates from the catabolism of Reducing Sugars (glucose and fructose), pyruvic acid, malic acid and citric acid, as already discussed for Example 3.

Fig. 17A shows the change of Indole-3-Lactic Acid in the tobacco material of Example 4 (VG-CH). The content of the Indole-3-Lactic is indicated in micrograms per gram. The graph shows a considerable increase in the content of Indole-3-Lactic Acid in the fermented tobacco material. The above indicated data for Indole-3-Lactic Acid have been obtained using Ultra Performance Liquid Chromatography polar and lipid positive and GC-MS; samples of non-fermented tobacco material (VG-NFHS CH VG-19-20-NF) and fermented tobacco

material (HS CH VG-19-20-F) subjected to a measurement in polar negative ionization mode. The samples were measured with a Waters ACQUITY Reversed Phase Ultra Performance Liquid Chromatography (RP-UPLC) coupled to a Thermo-Fisher Exactive mass spectrometer which consists of an ElectroSpray Ionization source (ESI) and an Orbitrap mass analyzer as well as with an Agilent Technologies mass spectrometer which consists of an Electron Impact ionization source (EI) and a Time of Flight (TOF) mass analyzer. UPLC-MS measurements of the aqueous phase enabled the detection of the polar and semi-polar primary and secondary metabolites, and the organic phase the detection of the lipid and lipophilic content. GC-MS measurements allow the analyses of the primary metabolites.

Sample Preparation

The sample preparation was performed according to metaSysX standard procedure, a modified protocol from Salem et al. (Salem *et al., Plant.Methods.* **2016** *45* (12)). 20 (+- 2) milligrams of ground material was used for metabolite extraction. Samples are extracted with MTBE:MetOH:H2O two-phase extraction method. Total (650 µl) organic phase is collected and dried down for LC-MS lipids measurement. 450 µl of polar phase is collected and dried down for LC-MS polar metabolites measurements and 150 µl polar phase is dried down and derivatized for GC-MS measurement.

Standard curve preparation

Stocks of 2 milligrams/ml of indole-3-lactic were solved in water. Standard mixes were prepared in a following concentrations: 10, 5, 2, 1, 0.5, 0.25, 0.125, 0.062 µg/ml . Further, 200 µl of standard mixes were dried down and subjected to the same extraction procedure as samples (“ex”) or directly subjected to LC-MS analysis or dried down, derivatized and analysed by GC-MS (no_ex).The extraction procedure for standard and samples was identical with all identical volumes

Calculation of the absolute content

Compounds concentrations were calculated based on extracted standard curves where all points (average of technical replicates) were taken for calculation and are presented as microgram per milligrams of the sample weight.

These data are reported in the table 3 reported below. This table shows he content of Indole-3-Lactic Acid of the diluted samples (standard), of the samples of the treated tobacco material of Example 4, (VG_IDC_AF), of the non-fermented tobacco material of Example 4 (VG_IDC_BF) and from different tobacco material non subjected to a fermentation process (K326_75, TN90_110, K326_G, K326_110, TN90_G, K326_cured, TN90_75)

Peak.ID	Mode	Analite	mz_mean	Adduct	Intensity	Concentration	Units	Sample

PN_9494	PN	Indole-3-Lactic Acid	204.0656671	[M-H]-	481151684	10	µg/ml	Standard
PN_9494	PN	Indole-3-Lactic Acid	204.0656671	[M-H]-	273727852	5	µg/ml	Standard
PN_9494	PN	Indole-3-Lactic Acid	204.0656671	[M-H]-	100375241.5	2	µg/ml	Standard
PN_9494	PN	Indole-3-Lactic Acid	204.0656671	[M-H]-	46290925	1	µg/ml	Standard
PN_9494	PN	Indole-3-Lactic Acid	204.0656671	[M-H]-	23189084.5	0.5	µg/ml	Standard
PN_9494	PN	Indole-3-Lactic Acid	204.0656671	[M-H]-	11308841.5	0.25	µg/ml	Standard
PN_9494	PN	Indole-3-Lactic Acid	204.0656671	[M-H]-	5366675	0.125	µg/ml	Standard
PN_9494	PN	Indole-3-Lactic Acid	204.0656671	[M-H]-	2644875.5	0.0625	µg/ml	Standard
PN_9494	PN	Indole-3-Lactic Acid	204.0656671	[M-H]-	304903692.5	10	µg/ml	Standard extracted
PN_9494	PN	Indole-3-Lactic Acid	204.0656671	[M-H]-	137499497	5	µg/ml	Standard extracted
PN_9494	PN	Indole-3-Lactic Acid	204.0656671	[M-H]-	40069896	2	µg/ml	Standard extracted
PN_9494	PN	Indole-3-Lactic Acid	204.0656671	[M-H]-	23274504.5	1	µg/ml	Standard extracted
PN_9494	PN	Indole-3-Lactic Acid	204.0656671	[M-H]-	11163531	0.5	µg/ml	Standard extracted
PN_9494	PN	Indole-3-Lactic Acid	204.0656671	[M-H]-	5869694.5	0.25	µg/ml	Standard extracted
PN_9494	PN	Indole-3-Lactic Acid	204.0656671	[M-H]-	2629402	0.125	µg/ml	Standard extracted
PN_9494	PN	Indole-3-Lactic Acid	204.0656671	[M-H]-	1247621.5	0.0625	µg/ml	Standard extracted
PN_9494	PN	Indole-3-Lactic Acid	204.0656671	[M-H]-	488683	0.000153746	µg/ml	TN90_cured
PN_9494	PN	Indole-3-Lactic Acid	204.0656671	[M-H]-	NA	NA	µg/mg	K326_75
PN_9494	PN	Indole-3-Lactic Acid	204.0656671	[M-H]-	NA	NA	µg/mg	TN90_110

PN_9494	PN	Indole-3-Lactic Acid	204.0656671	[M-H]-	NA	NA	µg/mg	K326_G
PN_9494	PN	Indole-3-Lactic Acid	204.0656671	[M-H]-	NA	NA	µg/mg	K326_110
PN_9494	PN	Indole-3-Lactic Acid	204.0656671	[M-H]-	NA	NA	µg/mg	TN90_G
PN_9494	PN	Indole-3-Lactic Acid	204.0656671	[M-H]-	174702	5.83E-05	µg/mg	K326_cured
PN_9494	PN	Indole-3-Lactic Acid	204.0656671	[M-H]-	NA	NA	µg/mg	TN90_75
PN_9494	PN	Indole-3-Lactic Acid	204.0656671	[M-H]-	8009875	0.002520006	µg/mg	VG_IDC_AF
PN_9494	PN	Indole-3-Lactic Acid	204.0656671	[M-H]-	339777	0.000113988	µg/mg	VG_IDC_BF

C-MS Measurements (Hydrophilic and Lipophilic Analytes)

The samples were measured with a Waters ACQUITY Reversed Phase Ultra Performance Liquid Chromatography (RP-UPLC) coupled to a Thermo-Fisher Exactive mass spectrometer. C8 and C18 columns were used for the lipophilic and the hydrophilic measurements, respectively. Chromatograms were recorded in Full Scan MS mode (Mass Range [100-1500]). All mass spectra were acquired in positive and negative ionization modes.

LC-MS Data Processing (Hydrophilic and Lipophilic Analytes)

Extraction of the LC-MS data was accomplished with the software PeakShaper (metaSysX GmbH). Alignment and filtration of the LC-MS data were completed using in-house software. After extraction from the chromatograms, the data is processed, aligned and filtered for redundant peaks. The alignment of the extracted data from each chromatogram was performed according to the criteria that a feature had to be present in all replicates of at least one of the groups. At this stage, the average RT and m/z values are given to the features. The alignment was performed for each type of measurements independently.

LC-MS Data Annotation (HydrophilicLipophilic and Analytes)

The in-house metaSysX database of chemical compounds was used to match features detected in the LC-MS lipophilic platform. The annotation of the compounds of interest was performed by matching with the MSX datatbase and confirmed by measured standards.

The metaSysX (MSX) database

The metaSysX in-house database contains mass-to-charge ratio and retention time information of 7500 reference compounds available as pure compounds and measured in the same chromatographic and spectrometric conditions as the measured samples. In addition, 1500 lipids and sugar esters putatively annotated based on the precursor m/z, fragmentation spectrum and elution patterns. The matching criteria for the DGDGs annotations were 5 parts per million and 0.085 minutes deviation from the reference compounds mass-to-charge ratio and retention time, respectively.

GC-MS Measurements

The samples were measured on an Agilent Technologies GC coupled to a Leco Pegasus HT mass spectrometer which consists of an EI ionization source and a TOF mass analyzer.

Column: 30 meters DB35; Starting temp: 85° degree Celsius for 2 minute; Gradient: 15° degree Celsius per min up to 360 ° degree Celsius.

GC-MS Data Processing and Annotation

NetCDF files that were exported from the Leco Pegasus software were imported to "R". The Bioconductor package TargetSearch[3]was used to transform retention time to retention index (RI), to align the chromatograms, to extract the peaks, and to annotate them.

The analysis performed on the samples indicates that the content of Indole-3-lactic in the fermented tobacco material (VG_IDC_AF) is 0.002520006 micrograms per milligrams; and the content of Indole-3-lactic in the fermented tobacco material (VG_IDC_BF) is 0.000113988 micrograms per milligrams. These data are reported in fig. 17A

Comparing the results obtained in the above discussed experiments, it is evident that the chemical analyses confirm that Lactic Acid massively increased after anaerobic fermentation in all the experiments conducted. Fully fermented tobacco material contained between about 50 milligrams per gram and about 100 milligrams per gram of Lactic Acid, whereas less than 4 milligrams per gram can be found in non-fermented tobacco. Data of Lactic Acid for different types of tobacco are reported in Table 2 in which Rajangan (RAJ) tobacco RAJ ID KS-18-19, Hand stripped (HS) dark tobacco HS ID KS-18-19, Hand stripped Virginia tobacco HS ID VG-19-20, and Hand stripped Virginia tobacco HS CH VG-19-20.

Rajangan (RAJ) tobacco ID KS-18-19 indicates the tobacco material of Example 2, HS ID KS-18-19 indicates the tobacco material of Example 1, HS ID VG-19-20 indicates the tobacco material of Example 3 and HS CH VG-19-20 indicates the tobacco material of Example 4.

Samples	Lactic Acid forms	Data set	sd	Replicate	sd

RAJ ID KS-18-19-NF	D-LACTIC ACID	378	64	276	47
	TOTAL LACTIC ACID	1578	210	1346	186
	L-LACTIC ACID	1200	200	1070	180
RAJ ID KS-18-19-F	D-LACTIC ACID	14700	2500	14800	2500
	TOTAL LACTIC ACID	86700	12300	87800	12300
	L-LACTIC ACID	72000	12000	73000	12000
HS ID KS-18-19-NF	D-LACTIC ACID	292	50	276	47
	TOTAL LACTIC ACID	1202	168	1246	176
	L-LACTIC ACID	910	160	970	170
HS ID KS-18-19-F	D-LACTIC ACID	15200	2600	16000	2700
	TOTAL LACTIC ACID	79200	11300	80000	11300
	L-LACTIC ACID	64000	11000	64000	11000
HS ID VG-19-20-NF	D-LACTIC ACID	630	110	700	120
	TOTAL LACTIC ACID	2260	290	2320	300
	L-LACTIC ACID	1630	270	1620	280
HS ID VG-19-20-F	D-LACTIC ACID	5940	1000	5820	980
	TOTAL LACTIC ACID	22400	3000	22120	2870
	L-LACTIC ACID	16500	2800	16300	2700
HS CH VG-19-20-NF	D-LACTIC ACID	1110	190	950	160
	TOTAL LACTIC ACID	3190	400	2690	340
	L-LACTIC ACID	2080	350	1740	300
HS CH VG-19-20-F	D-LACTIC ACID	10800	1800	12700	2200
	TOTAL LACTIC ACID	68700	10000	68600	9700
	L-LACTIC ACID	57900	9800	55900	9400

TABLE 4

Measurements of D-Lactic Acid and L-Lactic Acid (milligrams/kilograms DW) in the non-fermented compared to fermented samples, RAJ ID KS-18-19-NF compared to RAJ ID KS-18-19-F, HS ID KS-18-19-NF compared to HS ID KS-18-19-F; HS ID VG-19-20-NF compared to HS ID VG-19-20-F and HS CH VG-19-20-NF compared to HS CH VG-19-20-F

In Table 4 it is indicated the quantity of Lactic Acid in the fermented (F) and non-fermented (NF) tobacco material. The quantity of Lactic Acid is indicated as milligram per Kilogram with reference to the dry weight of the tobacco material. The data show that all the tobacco materials have an increase of the content of Lactic Acid after fermentation. All the tobacco samples have similar amount of Lactic Acid, thereby indicating that both fast-cured cut-filler with mid-rib tobacco and the corresponding hand-stripped cured material reach similar levels of Lactic Acid. Virginia tobacco reach similar level of Lactic Acid compared to Dark tobacco (Compare HS CH VG-19-20-F [68.7 milligrams per gram]with HS ID KS-18-19-F [79.2 milligrams per gram]).

This confirms that tobacco fermentation can be performed with different tobacco types, i.e Dark and Virginia tobacco, sugars and amino acids being a source of carbon and nitrogen for the Lactic Acid bacteria. Even if tests have not been performed with Burley and Oriental tobacco, nonetheless Oriental tobacco has a composition that is often close to that Dark tobacco with usually less alkaloids which are nonetheless not impacted by the anaerobic fermentation process.

Most of the Lactic Acid measured in fermented tobacco is the enantiomer L-Lactic Acid, which is in line with the biochemical pathway for anaerobic bacteria. However, some D-Lactic Acid is also produced. Compared to L-lactic-acid, D-Lactic Acid can be toxic to human, i.e the LD50 value level per orally poisoned rats is around 4.5 g/kilograms (Pohanka, 2020). However, D- and L-Lactic Acid are non-volatile, and therefore not transfer into aerosol.

Moreover tobacco it has been measured the fermentation Ratio for the tobacco material of examples 1-2 and 4-5 and the data are collected in Table 4 and 5 reported below

The tobacco fermentation ratio is given by the following formula: $Fr= (F_{LA}/NF_{LA}): (F_{RS}/NF_{RS})$ wherein:

Fr = Fermentation Ration; F_{LA}= content of Lactic Acid in the fermented tobacco material; NF_{LA} = initial content of Lactic Acid in the tobacco material; F_{RS} = content of Reducing Sugars in the fermented tobacco material; NF_{RS} = initial content of Reducing Sugars in the tobacco material.

	RS			LACTIC ACID		
	NF	F	**F/NF**	NF	F	**F/NF**
VG-ID	183.3	74.1	**0.4**	2.3	22.4	**9.7**
HS-ID	102.8	18.9	**0.2**	1.2	79.2	**66.0**
RJ-ID	106.0	25.0	**0.2**	1.7	86.7	**51.0**
VG-CH	18.9	1.0	**0.1**	3.2	68.7	**21.5**

Table 5. Fermentation ratio for Reducing Sugars (RS) and Lactic Acid, in which VG-ID indicates the tobacco material of Experiment 3, HS-ID indicates the tobacco material of Experiment 1, RJ-ID indicates the tobacco material of Experiment 2, VG-CH indicates the tobacco material of Experiment 4.

	F/NF(LA):F/NF(RS)
VG-ID	24
HS-ID	330
RJ-ID	255
VG-CH	215

Table 6. Fermentation index: ratio between F/NF Lactic Acid (LA) and F/NF RS

The tobacco fermentation Index gives a further indication about the fermentation of the tobacco material. The tobacco fermentation ration allows monitoring the level of fermentation in a tobacco sample. As shown in Table 5, the fermentation ration increases considerably during the fermentation. Therefore the fermentation ration is a very efficient indicator of the fermentation of the tobacco material.

In conclusion, metabolomic data suggests that the compounds generated during tobacco fermentation, namely Lactic Acid, indole-Lactic Acid, caffeic acid and quinic acid, are not directly linked to tobacco types, since they are produced with both Dark or Flue-cured tobacco matrices. However, they certainly may quantitatively change regarding the previous abundance of the substrates compounds found in the starting tobacco leaf material. Therefore anaerobic fermentation is a process applicable to different tobacco types and material and causes a change in the content of some substances in the tobacco material independently from the type of tobacco material.

Some substances may be used as indicator for the degree of fermentation of the tobacco material and these substances may be used as reliable indication of the degree of fermentation.

The use of many indicators and for example the correlation between the colour changes, the RS consumption and Lactic Acid production by the anaerobic bacteria attest of the degree of fermentation of the tobacco material and give a very reliable indication of the degree of fermentation.

Moreover the anaerobic process does not negatively impact the content of alkaloids of the tobacco material.

The experiments conducted lead to define that the following conditions may positively impact the fermentation increasing the efficiency of the process:

- Precondition hand-stripped leaf material with water to reach a final humidity of about comprised between about 40 percent by weight and 60 percent by weight indicated as Relative Humidity;
- Fill a close barrel, a tank or a container (barrel) with preconditioned strips to reach a capacity of about 100 kilograms;
- Maintain a relative humidity comprised between about 30 percent and 50 percent by weight with water in the closed container during the fermentation process;
- perform the fermentation at a temperature of about 22 Celsius degree;
- maintain almost stable the temperature during the fermentation process;

- perform at least one turning, during the fermentation process unloading the tobacco material, mixing the tobacco material and reloading the tobacco material in the fermentation device, in order to homogenize the anaerobic fermentation process within the container
- apply a pressure comprised between about 0.5 kilograms per square centimeter and 1 kilogram per square centimeter during the fermentation process, except when turning tobacco.
- Dry fermented tobacco material in a two steps drying process
- Subjecting the tobacco material to a first drying step in which the tobacco material is subjected to a temperature of about 60 Celsius degree for a period of about 8 min to reach about 25 percent OV
- Subjecting the tobacco material to a second drying step at a temperature comprised between about 90 Celsius degree and 100 Celsius degree at a pressure of about 0.6 bar for a period of time of about 10 min to reach 10-15percent OV.

On the other hand, as depicted in figure 10, and as already observed in Example 1 and Example 2, Reducing Sugars were used as substrate by fermenting bacteria. Therefore about 60percent of the Reducing Sugars (RS) were oxidized during the 8 month of fermentation, moving from 18.3 percent (VG-BF) to 7.4 (VG-AF) percent in dry weight (DW). It is possible that a longer period of fermentation would lead to a higher percentage of RS degradation

The present invention is also directed to the following embodiments:

1. Method for treating tobacco material, the method comprising:
    - providing a tobacco material
    - fermenting the tobacco material to obtain fermented tobacco material, the fermenting step including:
        - ✓ incubating the tobacco material under anaerobic conditions;
        - ✓ stopping the fermentation when at least one of the following conditions is satisfied:
        - ✓ the content of Lactic Acid is more than 10 times, preferably more than 20 times, more preferably more than 50 times, more preferably more than 70 times, preferably more than 80 times an initial amount of Lactic Acid in the tobacco material,
        - ✓ the content of Reducing Sugars is lower than 0.5, preferably lower than 0.4, more preferably below 0.2, more preferably below 0.1 an initial amount or Reducing Sugars in the tobacco material,
        - ✓ the content of Indole-3 Lactic Acid is more than 5 times, preferably more than 10 times, preferably more than 20 times, an initial amount of Indole-3 Lactic Acid in the tobacco material,
        - ✓ the content of caffeic acid is more than 4 times, preferably more than 10 times, preferably more than 20 times, an initial amount of caffeic acid in the tobacco material.

- ✓ the content of quinic acid is more than 2 times, preferably more than 4 times, an initial amount of quinic acid in the tobacco material,
- ✓ the content of asparagine is lower than 0.5, preferably lower than 0.4, preferably lower than 0.3 an initial amount or asparagine in the tobacco material,
- ✓ the content of Glutamine is lower than 0.5, preferably lower than 0.4 an initial amount or Glutamine in the tobacco material,
- ✓ the content of L-Ornithine is more than 10 times, preferably more than 20 times, preferably more than 50 times, preferably more than 100 times an initial amount of L-Ornithine in the tobacco material,
- ✓ the content of L-Leucine is more than 2 times, preferably more than 4 times, an initial amount of L-Leucine in the tobacco material,
- ✓ the content of L-Lysine is more than 2 times, preferably more than 6 times, an initial amount of L-Lysine in the tobacco material,
- ✓ the fermentation index is more than 50, preferably more than 100, more preferably more than 250, more preferably more 400, wherein the fermentation index is obtained dividing the ratio between the content of Lactic Acid in the treated tobacco material and the content of Lactic Acid in the non-fermented tobacco material by the ratio between the content of Reducing Sugars in the treated tobacco material and the content of Reducing Sugars in the non-fermented tobacco material.

2. Method according to embodiment 1 and further comprises an initial measuring step for measuring the initial content of at least one Lactic Acid, or Reducing Sugars, or Indole-3 Lactic Acid, or quinic acid, or caffeic acid, or L-Ornithine, or Asparagine, or Glutamine, or L-Leucine, or L-Lysine, or fermentation index in the tobacco material.
3. Method according to embodiment 1 or 2 and further comprising a measuring step for measuring the content of at least one Lactic Acid, or Reducing Sugars, or Indole-3 Lactic Acid, or quinic acid, or caffeic acid, or L-Ornithine, or Asparagine, or Glutamine or L-Leucine, or L-Lysine, or fermentation index in the tobacco material during the fermenting step.
4. Method according to any one of embodiments 1 to 3, wherein if the tobacco material contains dark tobacco, it is provided for stopping the fermentation when at least one of the following conditions is satisfied: the content of 2,3 butanediol is more than 5 times, preferably more than 10 times, an initial amount of 2,3 butanediol in the tobacco material, or the content of diacetyl is more than 5 times, preferably more than 10 times, an initial amount of diacetyl in the tobacco material.
5. Method according to embodiment 4 and further comprising an initial measurement step for measuring the initial content of 2,3 butanediol or diacetyl in the tobacco material before the

fermentation so as to obtain an initial amount respectively of 2,3 butanediol or diacetyl in the tobacco material.

6. Method for treating tobacco material, the method comprising:
   - providing a tobacco material
   - fermenting the tobacco material to obtain fermented tobacco material, the fermenting step including:
   - incubating the tobacco material under anaerobic conditions, wherein during the fermenting step it is provided for applying a pressure to the tobacco material comprised between 1000 kilograms per square meter and 15000 kilograms per square meter, preferably between 3000 kilograms per square meter and 12000 kilograms per square meter, more preferably between 5000 kilograms per square meter and 10000 kilograms per square meter.
7. Method for treating tobacco material, the method comprising:
   - providing a tobacco material
   - fermenting the tobacco material to obtain fermented tobacco material, the fermenting step including:
   - incubating the tobacco material under anaerobic conditions, wherein it is provided for continuing the fermenting step for a fermentation time of at least 1 month, preferably at least 2 months, more preferably at least 4 months, more preferably at least 6 months, even more preferably at least 8 months, preferably at least 10 months, more preferably at least 12 months.
8. Method for treating tobacco material, the method comprising:
   - providing a tobacco material
   - fermenting the tobacco material to obtain fermented tobacco material, the fermenting step including:
   - incubating the tobacco material under anaerobic conditions, wherein during the fermenting step it is provided for keeping the temperature of the tobacco material comprised between 21 degrees Celsius and 35 degrees Celsius, preferably between 25 degrees Celsius and 31 degrees Celsius.
9. Tobacco material obtained according to the method of any one of embodiments 1-8.
10. Tobacco material comprising at least one of the following compounds:
    - Lactic Acid in an amount that is more than 10 times, preferably more than 20 times, more preferably more than 50 times, more preferably more than 70 times, more preferably more than 80 times, an initial amount of Lactic Acid in the tobacco material.

- Reducing Sugars in an amount that is lower than 0.5, preferably lower than 0.4, more preferably below 0.2, more preferably below 0.1 an initial amount or Reducing Sugars in the tobacco material,
- Indole-3 Lactic Acid in an amount that is more than 5 times, preferably more than 10 times, preferably more than 20 times, an initial amount of Indole-3 Lactic Acid in the tobacco material.
- caffeic acid in an amount that is more than 4 times, preferably more than 10 times, an initial amount of caffeic acid in the tobacco material,
- quinic acid in an amount that is more than 2 times, preferably more than 4 times, an initial amount of quinic acid in the tobacco material,
- asparagine in an amount that is lower than 0.5, preferably lower than 0.4, preferably lower than 0.3 an initial amount or asparagine in the tobacco material,
- Glutamine in an amount that is lower than 0.5, preferably lower than 0.4 an initial amount or Glutamine in the tobacco material,
- L-Ornithine is more than 10 times, preferably more than 50 times, preferably more than 100 times an initial amount of L-Ornithine in the tobacco material,
- L-Leucine in an amount that is more than 2 times, preferably more than 4 times, an initial amount of L-Leucine,
- L-Lysine in an amount that is more than 2 times, preferably more than 6 times, an initial amount of L-Lysine,
- a fermentation index is more than 50, preferably more than 100, more preferably more than 250, more preferably more 400, wherein the fermentation index is obtained dividing the ratio between the content of Lactic Acid in the tobacco material and the content of Lactic Acid in the non-fermented tobacco material by the ratio between the content of Reducing Sugars in the tobacco material and the content of Reducing Sugars in the non-fermented tobacco material.

11. Tobacco material according to the preceding embodiment, wherein said tobacco material is obtained by a process comprising fermenting the tobacco material to obtain treated tobacco material, including: incubating the tobacco material under anaerobic conditions.
12. Tobacco material comprising at least 20 milligrams per gram, preferably at least 50 milligrams per gram, more preferably at least 60 milligrams per gram of Lactic Acid in total dry weight basis.
13. Tobacco material comprising:
    - less than 3 percent of total Reducing Sugars in total dry weight basis, and/or
    - less than 300 milligrams per kilogram of asparagine in total dry weight basis.

14. Tobacco material comprising less than 70 milligrams per kilogram of glutamine in total dry weight basis.
15. Tobacco material comprising more than 10000 milligrams per kilogram in total dry weight basis of total free amino acids.
16. Tobacco material comprising at least 1 microgram per gram, preferably at least 2 micrograms per gram, more preferably at least 2.5 micrograms per gram of Indole-3 Lactic Acid in total dry weight basis.
17. Tobacco material according to any one of embodiments from 19 to 27, wherein the tobacco material is cured.
18. Tobacco material according to any one of embodiments from 19 to 28, wherein the tobacco material is grinded.
19. Aerosol generating article comprising a tobacco material containing between about 2.5 percent by weight in total dry weight basis and 100 percent by weight in total dry weight basis, preferably at least about 4 percent by weight in total dry weight basis , preferably at least about 10 percent by weight in total dry weight basis, preferably at least about 20 percent by weight in total dry weight basis of a the tobacco material according to any one of embodiments 9 to 18.
20. Aerosol generating article comprising a tobacco material containing at least 5 milligrams per gram, preferably 10 milligrams per gram in total dry weight basis of Lactic Acid.

CLAIMS

1. Method for treating tobacco material, the method comprising:

providing a tobacco material;

fermenting the tobacco material to obtain fermented tobacco material, the fermenting step including:

incubating the tobacco material under anaerobic conditions;

stopping the fermentation when at least one of the following conditions is satisfied:

- ✓ the content of Lactic Acid is more than 10 times, preferably more than 20 times, more preferably more than 50 times, more preferably more than 70 times, preferably more than 80 times an initial amount of Lactic Acid in the tobacco material,
- ✓ the content of Reducing Sugars is lower than 0.5, preferably lower than 0.4, more preferably below 0.2, more preferably below 0.1 an initial amount or Reducing Sugars in the tobacco material,
- ✓ the content of Indole-3 Lactic Acid is more than 5 times, preferably more than 10 times, preferably more than 20 times, an initial amount of Indole-3 Lactic Acid in the tobacco material,
- ✓ the content of caffeic acid is more than 4 times, preferably more than 10 times, preferably more than 20 times, an initial amount of caffeic acid in the tobacco material,
- ✓ the content of quinic acid is more than 2 times, preferably more than 4 times, an initial amount of quinic acid in the tobacco material,
- ✓ the content of asparagine is lower than 0.5, preferably lower than 0.4, preferably lower than 0.3 an initial amount or asparagine in the tobacco material,
- ✓ the content of Glutamine is lower than 0.5, preferably lower than 0.4 an initial amount or Glutamine in the tobacco material,
- ✓ the content of L-Ornithine is more than 10 times, preferably more than 20 times, preferably more than 50 times, preferably more than 100 times an initial amount of L-Ornithine in the tobacco material,
- ✓ the content of L-Leucine is more than 2 times, preferably more than 4 times, an initial amount of L-Leucine in the tobacco material,
- ✓ the content of L-Lysine is more than 2 times, preferably more than 6 times, an initial amount of L-Lysine in the tobacco material,
- ✓ the fermentation index is more than 50, preferably more than 100, more preferably more than 250, more preferably more 400, wherein the fermentation index is obtained dividing the ratio between the content of Lactic Acid in the treated

tobacco material and the content of Lactic Acid in the non-fermented tobacco material by the ratio between the content of Reducing Sugars in the treated tobacco material and the content of Reducing Sugars in the non-fermented tobacco material.

2. Method according to claim 1 and further comprising an initial measuring step for measuring the initial content of at least one Lactic Acid, or Reducing Sugars, or Indole-3 Lactic Acid, or quinic acid, or caffeic acid, or L-Ornithine, or Asparagine, or Glutamine, or L-Leucine, or L-Lysine, or fermentation index in the tobacco material.

3. Method according to claim 1 or 2 and further comprising a measuring step for measuring the content of at least one Lactic Acid, or Reducing Sugars, or Indole-3 Lactic Acid, or quinic acid, or caffeic acid, or L-Ornithine, or Asparagine, or Glutamine or L-Leucine, or L-Lysine, or fermentation index in the tobacco material during the fermenting step.

4. Method according to any one of claims 1 to 3, wherein if the tobacco material contains dark tobacco, it is provided for stopping the fermentation when at least one of the following conditions is satisfied: the content of 2,3 butanediol is more than 5 times, preferably more than 10 times, an initial amount of 2,3 butanediol in the tobacco material, or the content of diacetyl is more than 5 times, preferably more than 10 times, an initial amount of diacetyl in the tobacco material.

5. Method according to claim 4 and further comprising an initial measurement step for measuring the initial content of 2,3 butanediol or diacetyl in the tobacco material before the fermentation so as to obtain an initial amount respectively of 2,3 butanediol or diacetyl in the tobacco material.

6. Method according to claim 1, wherein during the fermenting step it is provided for applying a pressure to the tobacco material comprised between 1000 kilograms per square meter and 15000 kilograms per square meter, preferably between 3000 kilograms per square meter and 12000 kilograms per square meter, more preferably between 5000 kilograms per square meter and 10000 kilograms per square meter.

7. Method according to claim 1, wherein it is provided for continuing the fermenting step for a fermentation time of at least 1 month, preferably at least 2 months, more preferably at least 4 months, more preferably at least 6 months, even more preferably at least 8 months, preferably at least 10 months, more preferably at least 12 months.

8. Method according to claim 1, wherein during the fermenting step it is provided for keeping the temperature of the tobacco material comprised between 21 degrees Celsius and 35 degrees Celsius, preferably between 25 degrees Celsius and 31 degrees Celsius.

9. Tobacco material comprising:

a) at least one of the following compounds:

- Lactic Acid in an amount that is more than 10 times, preferably more than 20 times, more preferably more than 50 times, more preferably more than 70 times, more preferably more than 80 times, an initial amount of Lactic Acid in the tobacco material.
- Reducing Sugars in an amount that is lower than 0.5, preferably lower than 0.4, more preferably below 0.2, more preferably below 0.1 an initial amount or Reducing Sugars in the tobacco material,
- Indole-3 Lactic Acid in an amount that is more than 5 times, preferably more than 10 times, preferably more than 20 times, an initial amount of Indole-3 Lactic Acid in the tobacco material.
- caffeic acid in an amount that is more than 4 times, preferably more than 10 times, an initial amount of caffeic acid in the tobacco material,
- quinic acid in an amount that is more than 2 times, preferably more than 4 times, an initial amount of quinic acid in the tobacco material,
- asparagine in an amount that is lower than 0.5, preferably lower than 0.4, preferably lower than 0.3 an initial amount or asparagine in the tobacco material,
- Glutamine in an amount that is lower than 0.5, preferably lower than 0.4 an initial amount or Glutamine in the tobacco material,
- L-Ornithine is more than 10 times, preferably more than 50 times, preferably more than 100 times an initial amount of L-Ornithine in the tobacco material,
- L-Leucine in an amount that is more than 2 times, preferably more than 4 times, an initial amount of L-Leucine,
- L-Lysine in an amount that is more than 2 times, preferably more than 6 times, an initial amount of L-Lysine,
- a fermentation index is more than 50, preferably more than 100, more preferably more than 250, more preferably more 400, wherein the fermentation index is obtained dividing the ratio between the content of Lactic Acid in the tobacco material and the content of Lactic Acid in the non-fermented tobacco material by the ratio between the content of Reducing Sugars in the tobacco material and the content of Reducing Sugars in the non-fermented tobacco material; or

b) less than 300 milligrams per kilogram of asparagine in total dry weight basis; or

c) less than 70 milligrams per kilogram of glutamine in total dry weight basis; or

d) more than 10000 milligrams per kilogram in total dry weight basis of total free amino acids; or

e) at least 1 microgram per gram, preferably at least 2 micrograms per gram, more preferably at least 2.5 micrograms per gram of Indole-3 Lactic Acid in total dry weight basis.

10. Tobacco material according to the preceding claim, wherein said tobacco material is obtained by a process comprising fermenting the tobacco material to obtain treated tobacco material, including: incubating the tobacco material under anaerobic conditions.

11. Tobacco material according to claim 9 or 10, wherein the tobacco material is cured.

12. Tobacco material according to any one of claims 9 to 11, wherein the tobacco material is grinded.

13. Aerosol generating article comprising a tobacco material containing between about 2.5 percent by weight in total dry weight basis and 100 percent by weight in total dry weight basis, preferably at least about 4 percent by weight in total dry weight basis , preferably at least about 10 percent by weight in total dry weight basis, preferably at least about 20 percent by weight in total dry weight basis of a the tobacco material according to any one of claims 9 to 12.

FIG.2

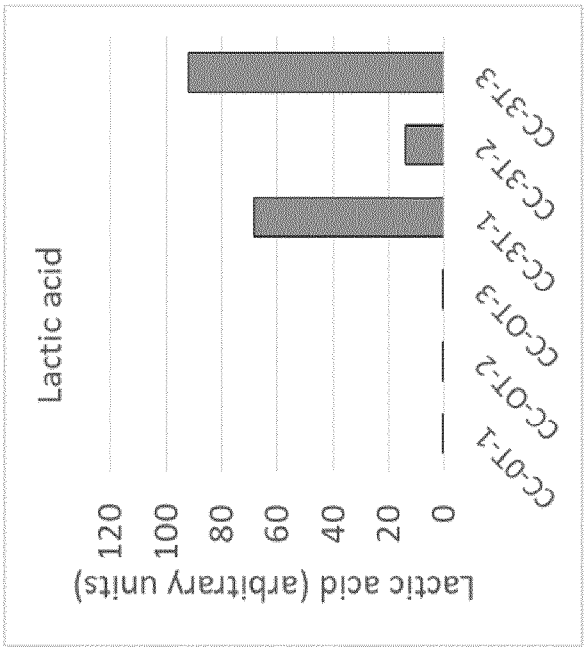


FIG.1

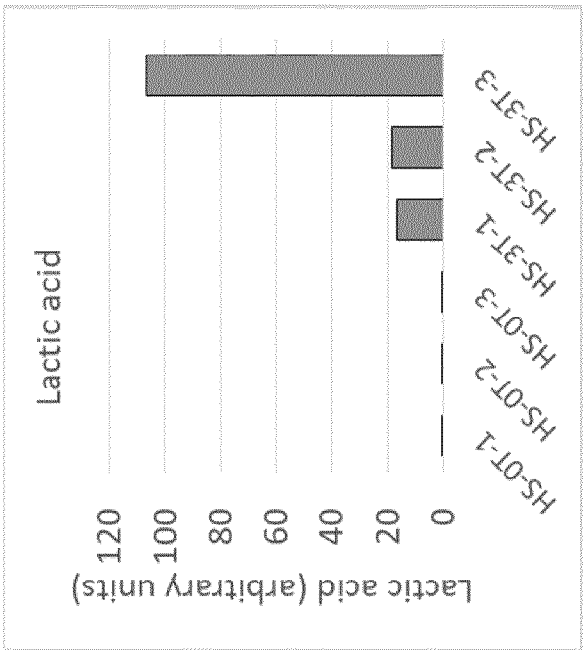


FIG.4

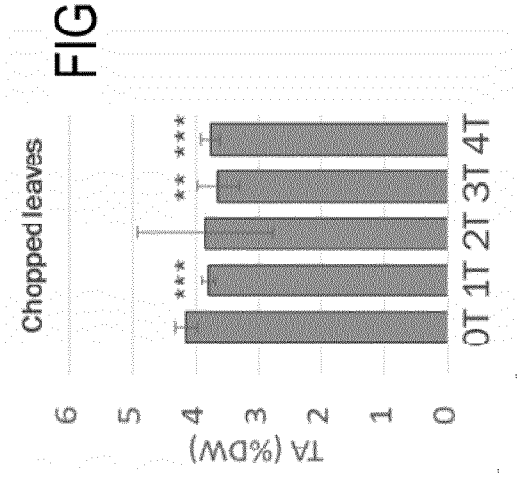


FIG.3

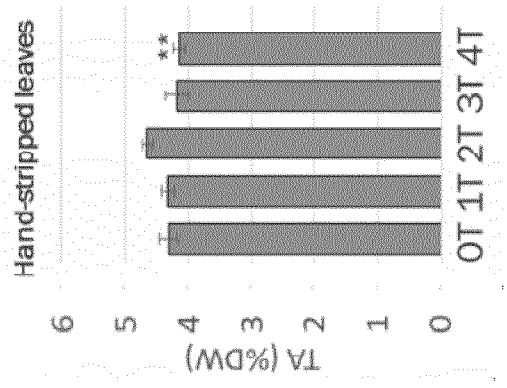


FIG.5

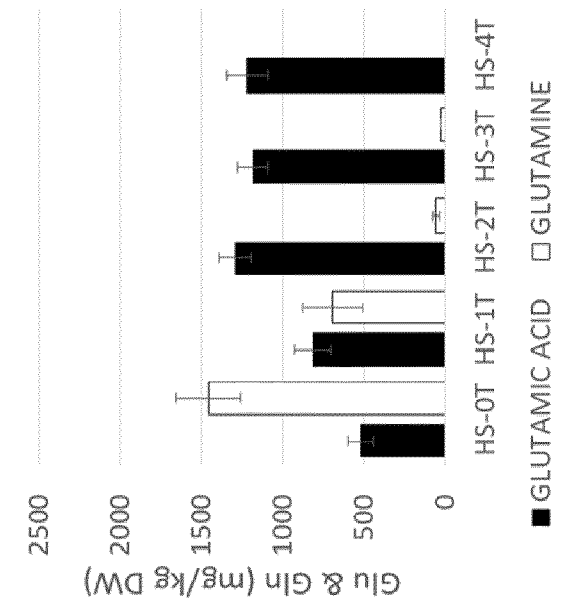


FIG.6

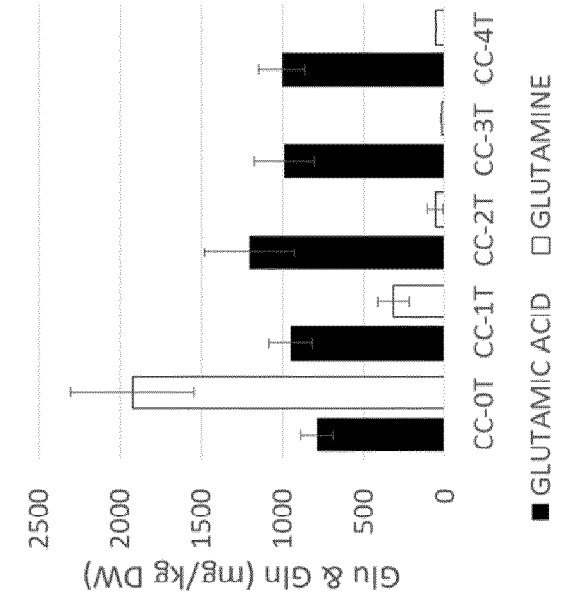


FIG.7

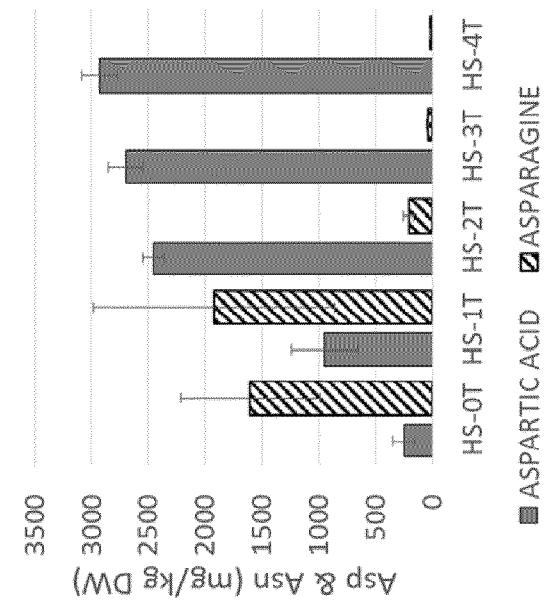
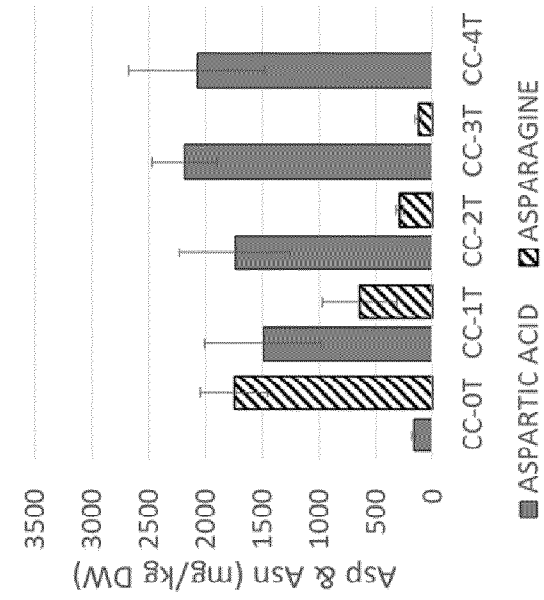


FIG.8



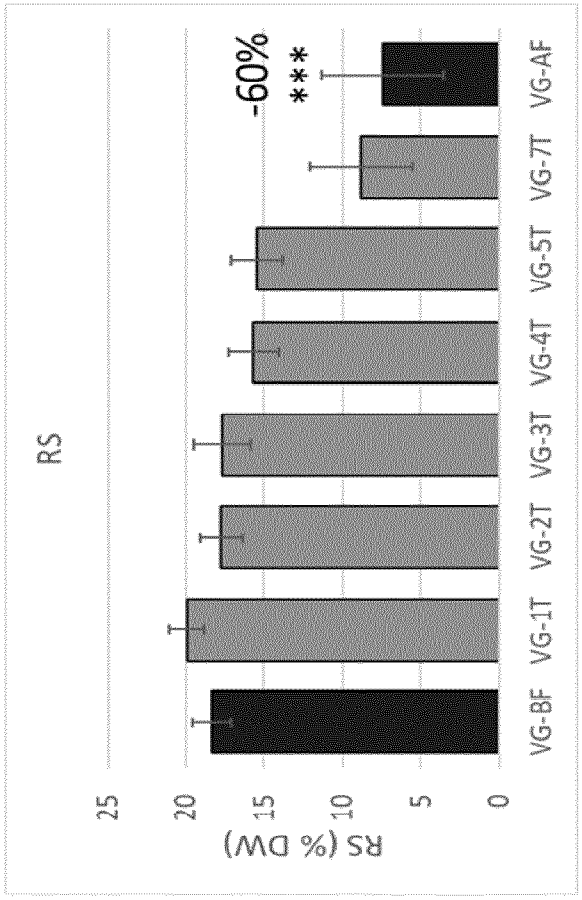

RS
RS (% DW)
25
20
15
10
5
0
-60%
***
VG-BF
VG-1T
VG-2T
VG-3T
VG-4T
VG-5T
VG-7T
VG-AF

FIG.10

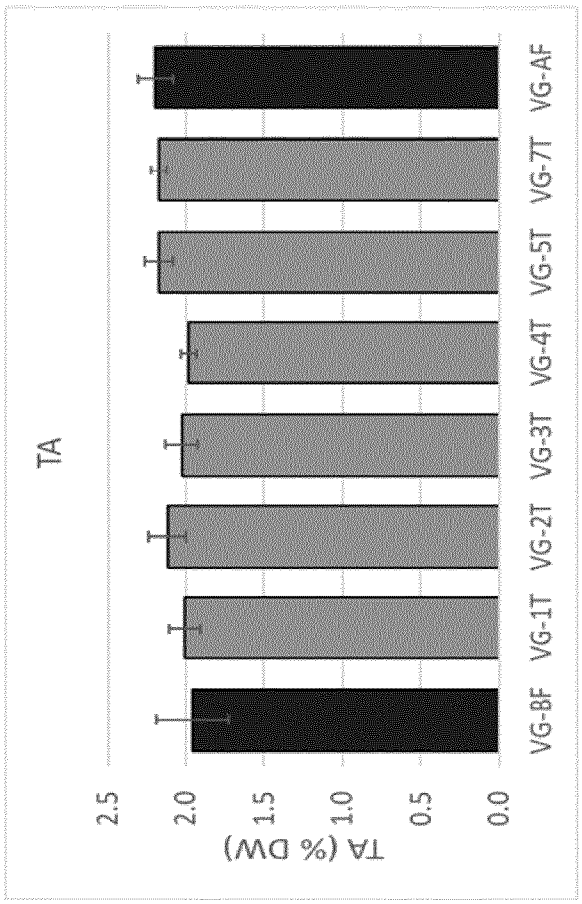

TA
TA (% DW)
2.5
2.0
1.5
1.0
0.5
0.0
VG-BF
VG-1T
VG-2T
VG-3T
VG-4T
VG-5T
VG-7T
VG-AF

FIG.9

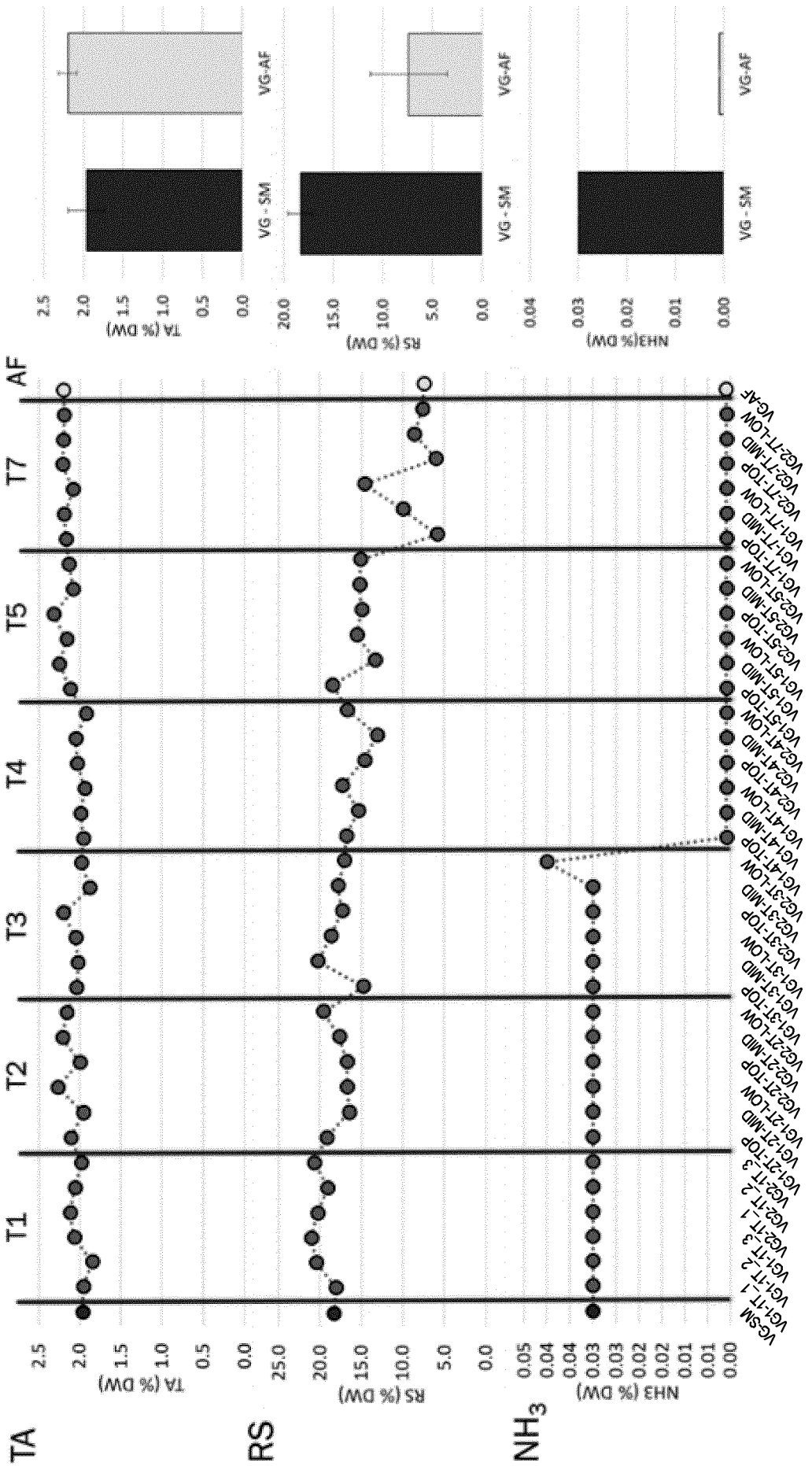

TA
RS
NH3
TA (% DW)
RS (% DW)
NH3 (% DW)
VG - SM
VG-AF
T1
T2
T3
T4
T5
T7
AF
VG-SM
VGi-1T-1
VGi-1T-2
VGi-1T-3
VG2-1T-1
VG2-1T-2
VG2-1T-3
VGi-2T-TOP
VGi-2T-MID
VGi-2T-LOW
VG2-2T-TOP
VG2-2T-MID
VG2-2T-LOW
VGi-3T-TOP
VGi-3T-MID
VGi-3T-LOW
VG2-3T-TOP
VG2-3T-MID
VG2-3T-LOW
VGi-4T-TOP
VGi-4T-MID
VGi-4T-LOW
VG2-4T-TOP
VG2-4T-MID
VG2-4T-LOW
VGi-5T-TOP
VGi-5T-MID
VGi-5T-LOW
VG2-5T-TOP
VG2-5T-MID
VG2-5T-LOW
VGi-7T-TOP
VGi-7T-MID
VGi-7T-LOW
VG2-7T-TOP
VG2-7T-MID
VG2-7T-LOW
VG-AF

FIG.11

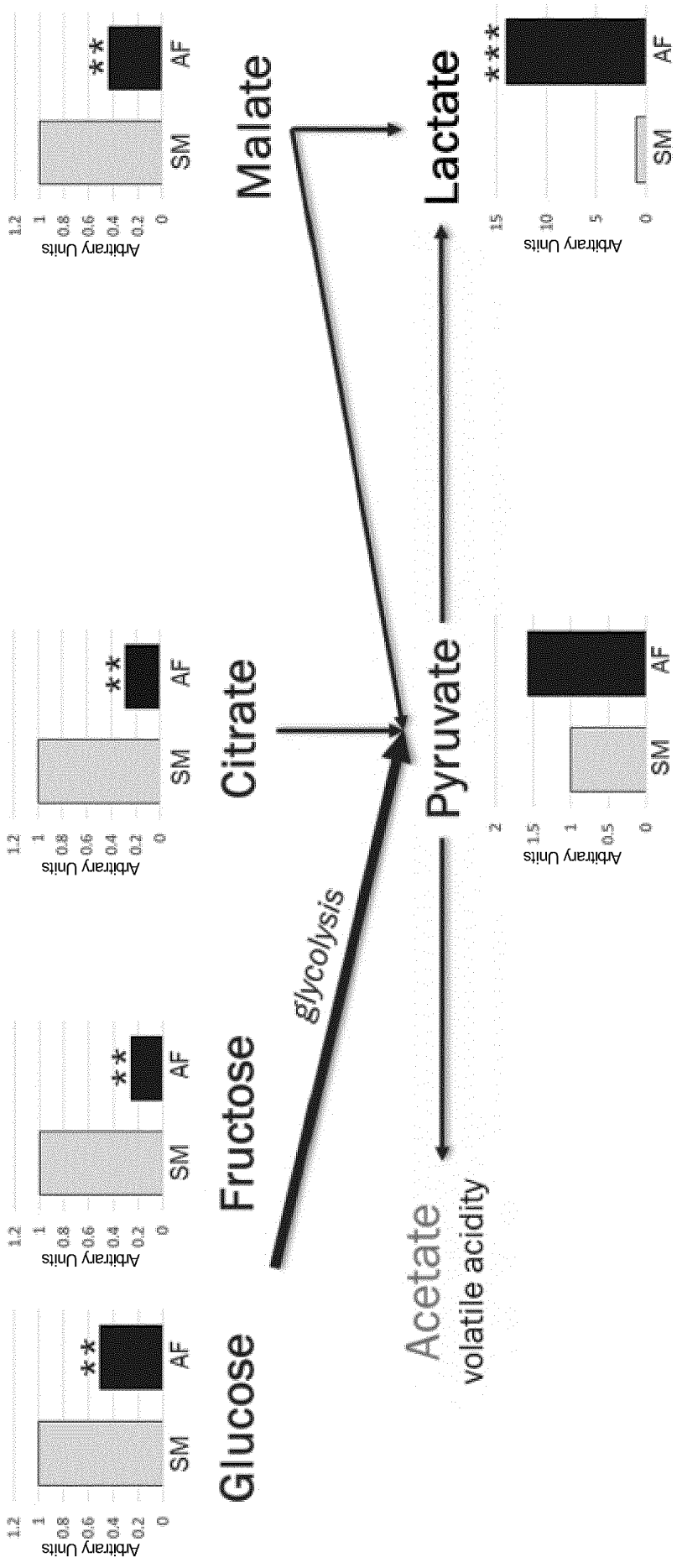

Glucose
Fructose
glycolysis
Citrate
Malate
Pyruvate
Lactate
Acetate
volatile acidity
Arbitrary Units
SM
AF
**
***

FIG.12

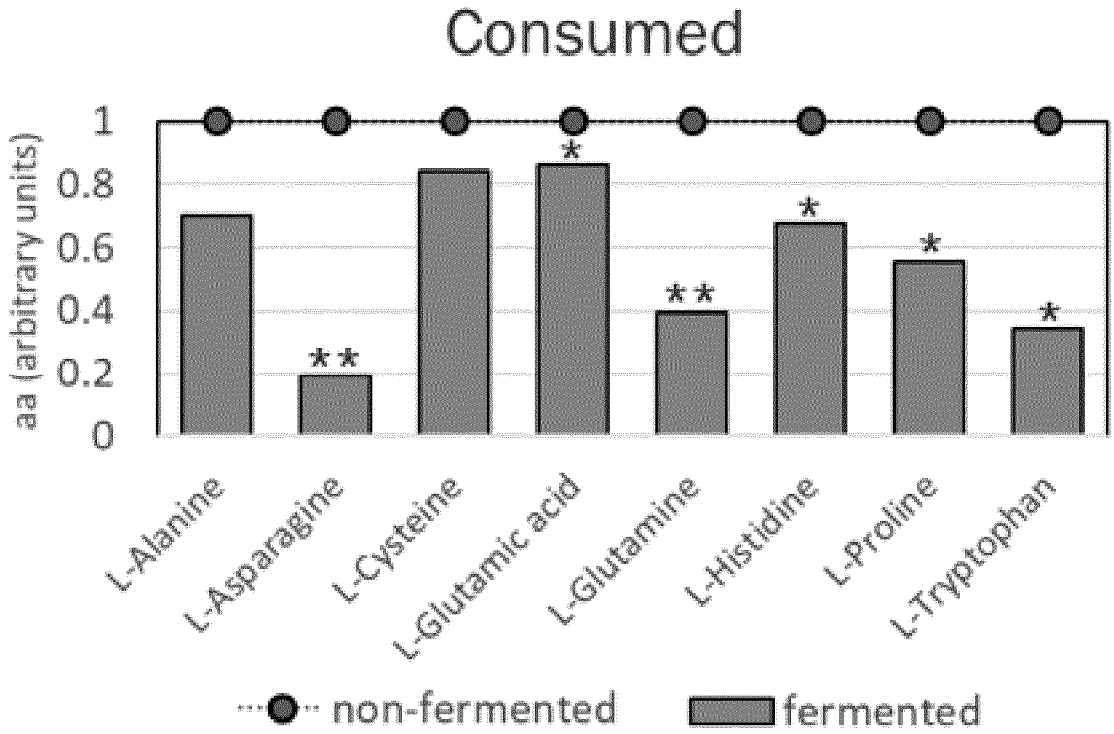


FIG.13A

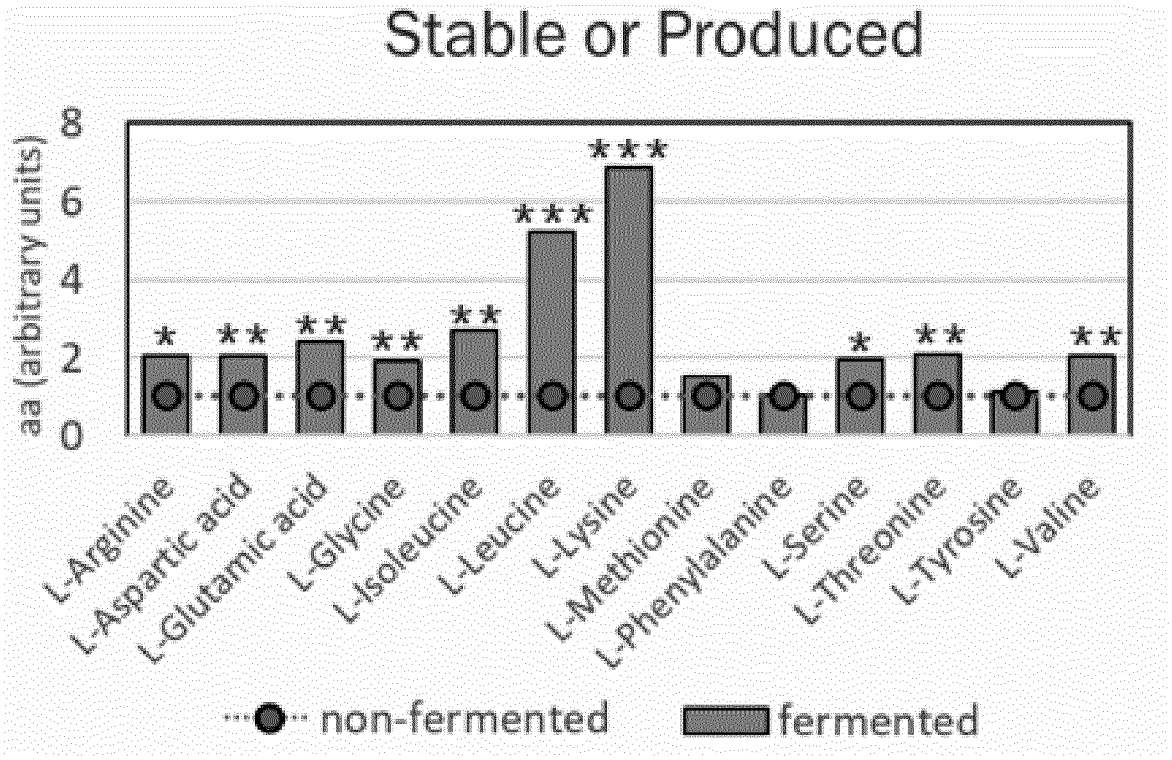


FIG.13B

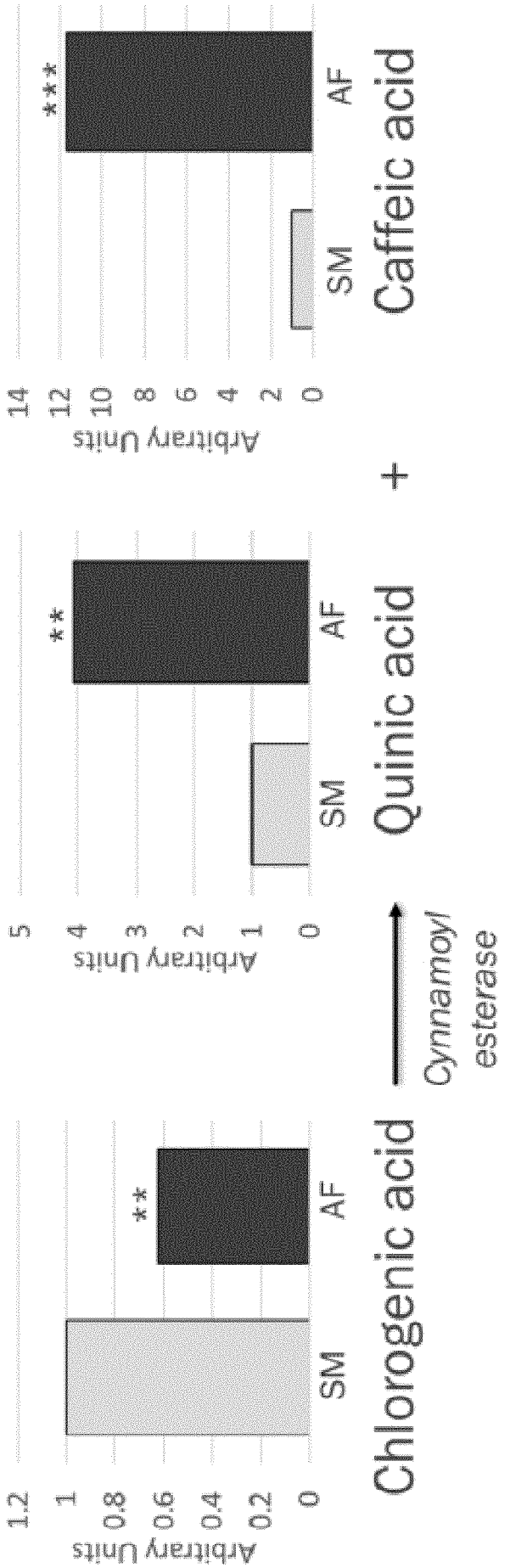

Arbitrary Units
0
0.2
0.4
0.6
0.8
1
1.2
**
SM
AF
Chlorogenic acid
Cynnamoyl esterase
Arbitrary Units
0
1
2
3
4
5
**
SM
AF
Quinic acid
+
Arbitrary Units
0
2
4
6
8
10
12
14
***
SM
AF
Caffeic acid

FIG.14

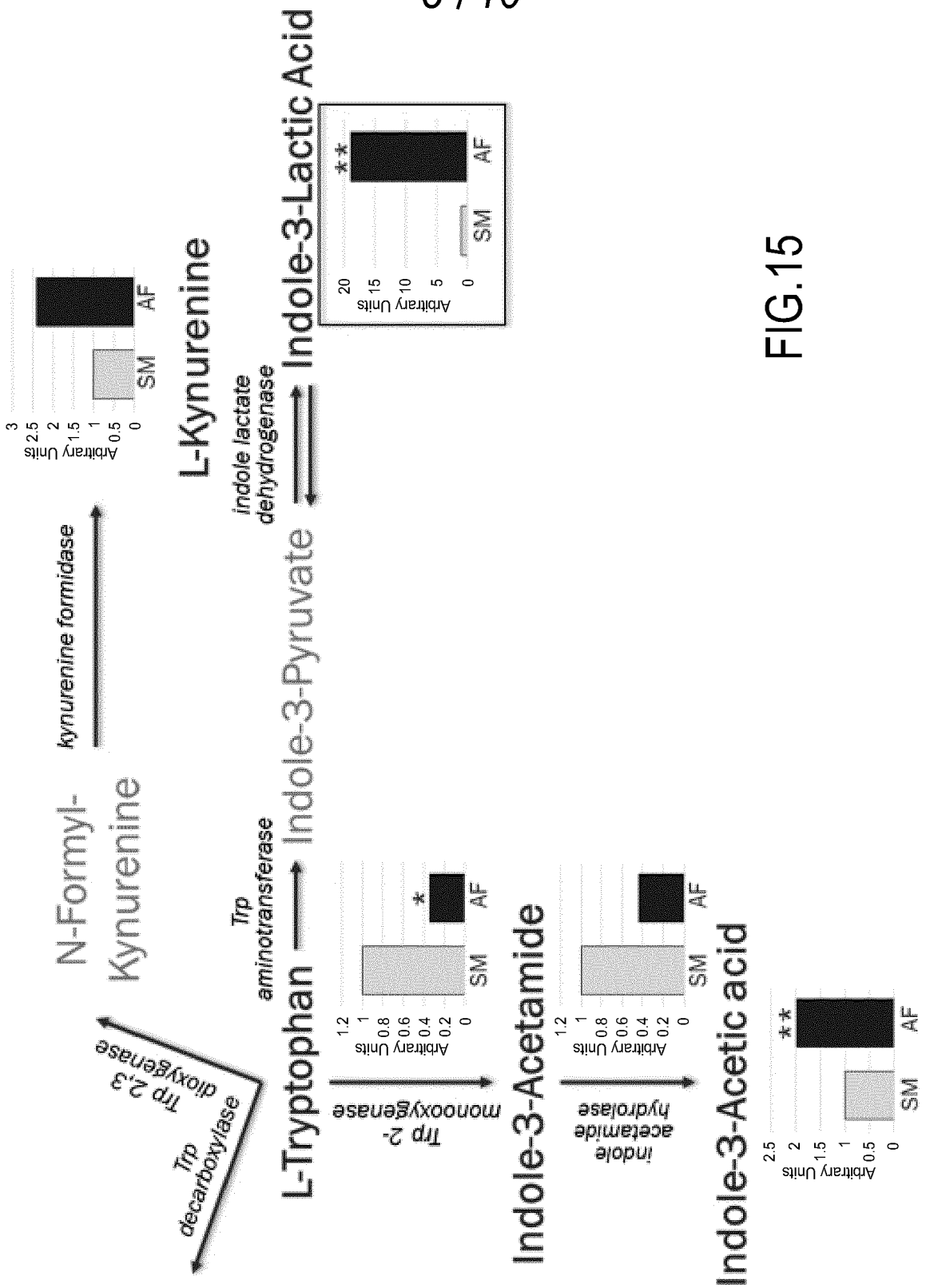

Tryptamine
Trp decarboxylase
Trp 2,3 dioxygenase
N-Formyl-Kynurenine
kynurenine formidase
L-Kynurenine
Trp aminotransferase
L-Tryptophan
Indole-3-Pyruvate
indole lactate dehydrogenase
Indole-3-Lactic Acid
Trp 2-monooxygenase
Indole-3-Acetamide
indole acetamide hydrolase
Indole-3-Acetic acid
Arbitrary Units
SM
AF
*
**

FIG.15

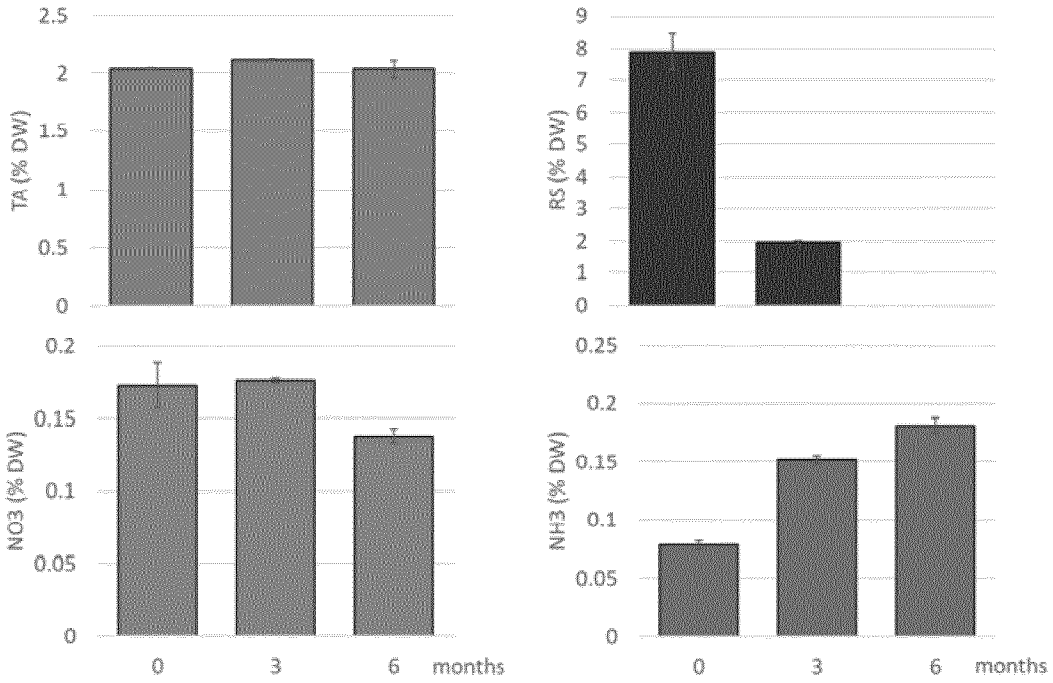


FIG.16

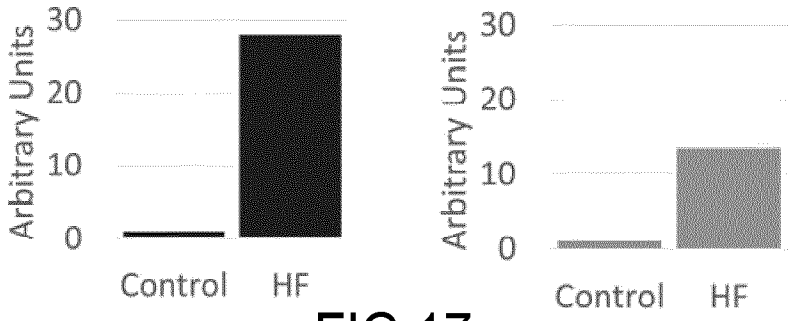


FIG.17

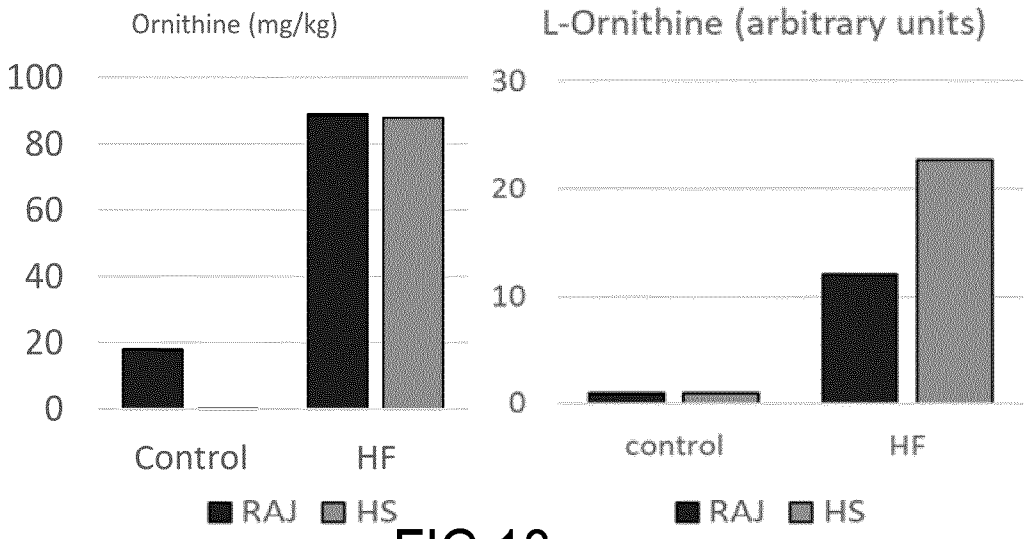


FIG.18

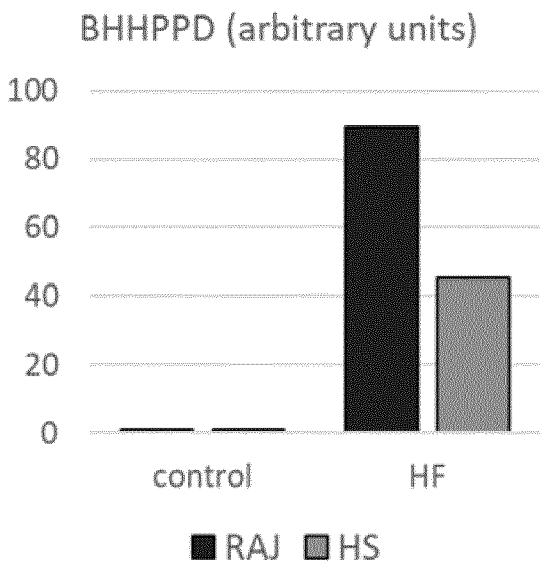


FIG.19

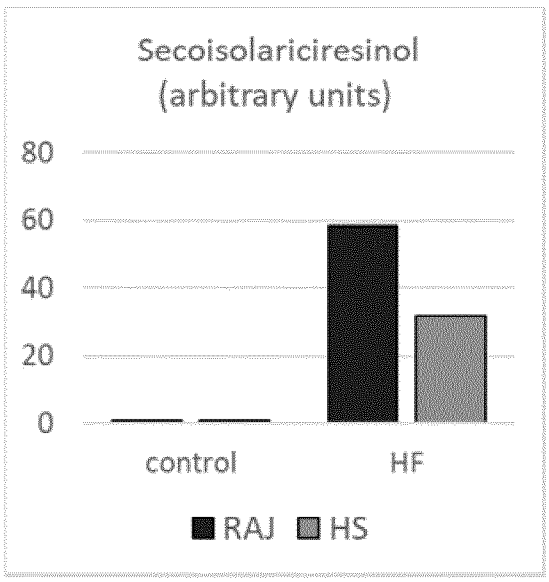


FIG.20

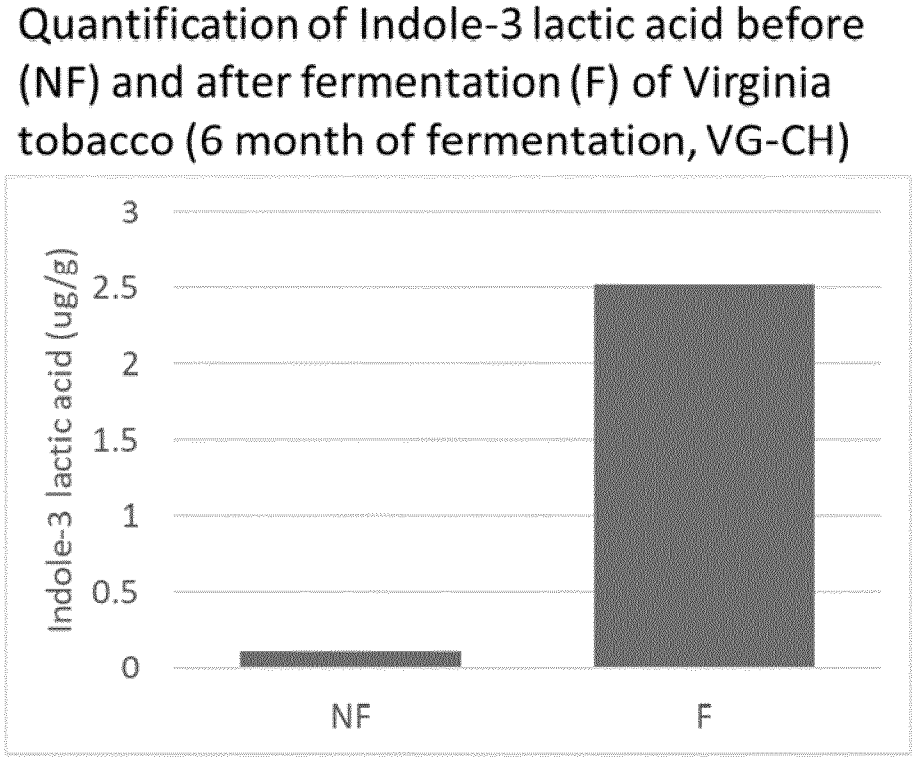


FIG.17A

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2022/080842

A. CLASSIFICATION OF SUBJECT MATTER
INV. A24B15/18 A24B15/30
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A24B

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, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GB 2 548 543 A (PHILIP MORRIS PRODUCTS SA [CH]) 27 September 2017 (2017-09-27) page 2, line 30; claims 1, 3 page 2, line 32 page 11, line 7 - line 14; example 2 page 15, line 31 - line 33	1-13
A	GB 2 542 623 A (PHILIP MORRIS PRODUCTS SA [CH]) 29 March 2017 (2017-03-29) page 23, line 12; claims 1, 11, 13 page 5, line 31 - line 33 page 4, line 28 - line 32 page 22, line 15 - line 17	1-13
A	CN 108 541 999 A (UNIV ZHENGZHOU LIGHT IND) 18 September 2018 (2018-09-18) claim 1; example 3	1-13

-/--

[x] Further documents are listed in the continuation of Box C.

[x] 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

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 March 2023	14/03/2023

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Picout, David

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2022/080842

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 3 070 098 A (TEMPEL EGGO J ET AL) 25 December 1962 (1962-12-25) the whole document	1-13
A	US 2021/186083 A1 (MARSHALL JERRY WAYNE [US] ET AL) 24 June 2021 (2021-06-24) the whole document	1-13

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2022/080842

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
GB 2548543 A	27-09-2017	NONE	
GB 2542623 A	29-03-2017	NONE	
CN 108541999 A	18-09-2018	NONE	
US 3070098 A	25-12-1962	NONE	
US 2021186083 A1	24-06-2021	NONE	